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STUDIES ON THE SEEDBORNE NATURE AND CONTROL OF *FUSARIUM* WILT OF BASIL

A Thesis Presented

by

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ABSTRACT

STUDIES ON THE SEEDBORNE NATURE AND CONTROL
OF *FUSARIUM* WILT OF BASIL

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The seedborne nature and control of the vascular wilt disease of basil (*Ocimum basilicum* L.), caused by *Fusarium oxysporum* f. sp. *basilicum* (Schlectend:Fr.) (*Fob*), was investigated. *Fob* was found to be a surface contaminant and perhaps an internal inhabitant of basil seed. Twenty-four commercial seed lots, originating from the U.S.A., Germany, Holland, and Italy, were tested for *Fusarium* contamination levels. Sixteen of the seed lots surveyed were contaminated with *Fusarium*, with levels of infestation ranging between 1%

and 26%. Pathogenicity tests demonstrated that twenty-five of the twenty-nine isolates obtained from contaminated seed caused wilt on basil. Vegetative compatibility grouping and RAPD-PCR analysis confirmed the isolates were *Fob*.

Six chemical, biological, and physical seed treatments were investigated for their potential to eliminate *Fob* from basil seed. Only a hot water treatment of 60°C or a combination of bleach (sodium hypochlorite) and hot water were able to completely eliminate *Fob* from seed. Assessing the success of the seed treatments by growing the plants out in the greenhouse was found to be more sensitive than culture plating.

Mycostop® soil drench treatments and the use of composted potting mixes were not effective in preventing *Fusarium* wilt of basil; although the latter practice demonstrated potential for delaying symptom expression.

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INTRODUCTION AND LITERATURE REVIEW

1.1 *Fusarium* Wilt of Basil

A vascular wilt disease of sweet basil, *Ocimum basilicum* L., Lamiaceae, caused by an unknown *Fusarium* species, was first reported in Russia in 1956 (Vergovskii, 1956; Kvartskhava, 1957). The symptoms were reported as longitudinal stripes on the stem, and chlorotic leaves which wilted and dropped prematurely. The causal agent was first called *Fusarium oxysporum* f. sp. *basilicum* (Schlechtend.:Fr.) by Dzidzariya (1968). The disease was later noted in Italy (Grasso, 1975), France (Mercier and Pionnat, 1982), the United States (Wick and Haviland, 1992), and, most recently, Australia (Vaughn, personal communication), Canada (Wick, personal communication), and Israel (Elmer, personal communication). Within the United States, the disease has occurred in California (Davis and Marshall, 1993), Colorado, Connecticut, Louisiana, Hawaii (Uchida and Kadooka, 1996), Maryland (Dutky and Wolkow, 1994), Massachusetts (Wick and Haviland, 1992), Michigan, Minnesota, New Mexico, New York, Oregon, and South Carolina (Keinath, 1993).

1.1.1 The host

Basil is an annual herb belonging to the Lamiaceae, or mint family. There are over 160 species of *Ocimum*, including *O. canum* (hoary basil), *O. gratissimum* (East Indian or tree basil), and *O. sanctum* (holy basil); although taxonomy remains confused due to the plant's polymorphic character and because it cross pollinates. Many subspecies, varieties, and forms of basil have been designated (Simon, 1984). Cultivated varieties of *O. basilicum* (sweet basil) include 'Minimum' (bush basil), 'Purpurascens' (purple basil), and 'Citriodora' (lemon basil) (Foster, 1993). The wild parentage of *O. basilicum* is unknown (Darrah, 1980).

The geographic origin of basil is also unknown, although it is believed to have been introduced into Greece during Alexander the Great's Asian campaign. Therefore, the plant may have been found in Persia, which is now Iran. It was brought to the Massachusetts Bay Colony in 1621. The Latin name is taken from the ancient Greek *okimon*, which means smell. There is much folklore attached to basil, some of which appears paradoxical. The plant is believed to signify love, fidelity, fertility, chastity, good health, grief, good fortune, or evil, depending on the time period,

religion, and civilization in question (Darrah, 1980). Basil possesses medicinal properties as well. The water and alcoholic extracts of *O. basilicum* leaves are said to produce strong antiulcer activities. The Chinese use basil to relieve stomach spasms and kidney ailments. Mexican-Americans and the Chinese use basil during childbirth to alleviate pain and to promote blood circulation (Foster, 1993). Native peoples utilize basil for the treatment of sore eyes, ears, and stuffy noses. Basil oils have been found to possess antimicrobial, fungistatic, nematicidal, and insecticidal properties (Foster, 1993).

Basil is also coveted for its diverse culinary potential and its fragrant essential oils. The aromatic leaves can be used as a flavoring or spice in sauces, stews, salad dressings, vegetables, poultry dishes, vinegars, and alcoholic beverages (Simon and Craker, 1984). The oils are used in perfumes, soaps, and shampoos (Simon, 1984). The essential oil from European sweet basil contains methylchavicol, linalool, and varying amounts of many other compounds. Concentrations of these chemicals vary depending upon the species, variety, time of harvest, soil, and weather conditions (Foster, 1993).

Sweet basil is an erect, branched, annual dicotyledon. The 'Italian' variety of sweet basil grows up to 100 cm in height, whereas *O. basilicum* 'Minimum' (bush basil) and *O. basilicum* 'Citriodora' (lemon basil) only reach 30 cm. The fragrant green leaves are smooth, oval, and acutely pointed with entire margins. The large leaves of the 'Italian' variety are 4 - 5 cm long. Flowers are arranged on racemes, which are 6 - 12 mm long, in whorls of six flowers. The flowers are white to purple in color. The black seeds are about 1.6 mm in diameter and produce mucilage when soaked in water.

Basil is cultivated extensively in France, Egypt, Hungary, Indonesia, Morocco, Greece, and the United States. In fact, basil is probably the most widely cultivated herb in American gardens (Foster, 1993). Basil is one of the easiest herbs to grow successfully. The seed can be sown indoors or directly in the garden as soon as the soil is warm enough. The seed germinates in 5 to 14 days, bloom begins 8 to 10 weeks after planting, and full bloom takes 12 to 14 weeks. Cultivated *Ocimum* is pollinated primarily by honeybees, and cross pollination is common (Darrah, 1980). The plant is susceptible to frost, favoring temperatures of

7 - 27C, and flourishes in full sun and well-watered, well-drained soil. The soil pH should be between 4.3 - 8.2.

Because of the antimicrobial and insecticidal properties of its essential oils, basil is thought to be relatively free of diseases. Horst (1979) reports that in parts of the United States, basil has been a host for root knot nematode (*Meloidogyne* spp.) and for bromegrass mosaic virus. Several fungal diseases have been reported in India, such as leaf blight caused by *Colletotrichum capsici* (Alam et al., 1980); scab, caused by *Elsinoe arxii* sp. *nov* (Sridhar and Ullasa, 1979); *Alternaria* leaf spot (Khadr and Abdel-Kader, 1974) and *Corynespora* leaf spot (Devi et al., 1979). Hungarian researchers have recognized viral and mycoplasmic diseases of basil (Gaborjanyi and Nagy, 1972; Horvath, 1979) as well as *Cercospora* blight caused by C. *ocimicola* (Upadhyay et al, 1976).

To achieve the best quality of harvest, the basil leaves should be picked from the main branches just above the suckers growing in the leaf nodes, just before the flowers develop (Ogden, 1990). The plants will subsequently produce new leaves for a more bountiful harvest. Commercial basil growers harvest the leaves by hand if the plants are

greenhouse or hydroponically grown. The seeds of rarer varieties of basil are hand harvested. Machines are used to harvest the seeds of commercially important varieties, either by cutting and threshing separately, or by the direct combine method. In the first method, the plant tops are cut and left on the ground to dry, then threshed either by hand or through a threshing machine. The direct combine method integrates the two actions utilizing one machine; however, the seeds must be already be dry enough to harvest.

1.1.2 The pathogen

Fusarium oxysporum was first named as a species by Schlectendahl (1824). The species included a group of fusaria in the section Elegans, named by Wollenweber and Reinking (1935), which mainly caused vascular wilt diseases. The species was further divided by Snyder and Hansen (1940) into formae speciales, a taxon which delineated host specificity of the fungal strain rather than morphological differences. Snyder and Hansen recognized 25 formae speciales of *F. oxysporum*, which could be discerned through pathogenicity tests. However, they mistakenly assumed, as had other researchers at the time, that one forma specialis had only one host or host group upon which it is a parasite.

Accordingly, they named each form after the Latin name of the crop it was believed to exclusively parasitize. In fact, in 1940, the same year Snyder and Hansen's paper was published, it was reported that the *Fusarium* species which caused cotton wilt, *F. oxysporum* f. sp. *vasinfectum*, not only infected cotton but caused disease on plants in the Malvaceae, Solanaceae, and Leguminosae families as well (Armstrong and Armstrong, 1975). Two plant pathologists, George M. Armstrong and his wife, Joanne K. Armstrong, undertook an extensive study of all *Fusarium* wilt pathogens to see if other fusaria behaved likewise. They found, for example, *F. oxysporum* f. sp. *cassiae* causes wilt on not only *Cassia* tora but on alfalfa, celery, chrysanthemum, cotton, lupine, and tobacco (Armstrong and Armstrong, 1975). The degree of pathogenicity was distinguishable on the different hosts, however, leading to the concept of primary and secondary hosts. If the virulence of a forma specialis for one host remained stable for a time, but a reduction or loss of pathogenicity occurred for another host, the first host was considered primary and the other the secondary host (Armstrong and Armstrong, 1975). This was not found to be so with alfalfa, where the disease complexes were found to

be equivalent with three separate formae speciales (f. sp. *cassiae*, f. sp. *vasinfectum*, and f. sp. *medicaginis*).

There are not only a multiplicity of hosts for some formae speciales, there are also a number of pathogenic races (fungal strains which differ in their degree of pathogenicity to a host) within some formae speciales. For example, *F. oxysporum* f. sp. *lycopersici* and f. sp. *melonis* each have three races, and f. sp. *pisi* and f. sp. *vasinfectum* each have five races. It was even decided to call *F. oxysporum* f. sp. *raphani* and f. sp. *mathioli*, races 2 and 3, respectively, of f. sp. *conglutinans*, which attacks cabbage (Armstrong and Armstrong, 1975).

There were a few formae speciales which did show true host specificity, including *fragariae*, *glycines*, *medicaginis*, and *vanillae* (Windels, 1992). There are also cases where a forma specialis infects a different host from its primary host, but does not cause disease. For example, f. sp. *batatas*, the sweet-potato wilt fungus, was found in cotton and Mexican clover, but did not cause wilt on these hosts (Armstrong and Armstrong, 1948).

The survival and proliferation of *F. oxysporum* is dependent upon several different structures. Although no

sexual stage is known for the fungus, it produces three types of asexual spores: chlamydospores, microconidia, and macroconidia (Marois, 1990). The chlamydospores are normally formed readily and profusely in culture, singly or in pairs. Microconidia are abundant, generally single-celled, oval to kidney-shaped, and produced only in false heads. Macroconidia are abundant, slightly sickle-shaped, thin-walled, and delicate, with an attenuated apical cell and a foot-shaped basal cell. The conidia are borne on unbranched or branched monophialides. On potato dextrose agar, fungal growth is rapid, producing white, aerial mycelium. Sclerotia, if present, are blue, and the sporodochia are cream to tan to orange (Nelson et al., 1983). *Fusarium oxysporum* can live as a pathogen or as a saprophyte, although it is a poor soil competitor; it can only colonize organic matter that has not already been colonized by other microorganisms. Many *F. oxysporum* strains need up to 100 propagules per gram of soil to cause disease.

The life cycle of *F. oxysporum* was summarized by Nelson (1981). The fungus is able to remain dormant as chlamydospores until it is stimulated to germinate, through

contact with either host or nonhost plant roots, or pieces of fresh, noncolonized plant debris. Most vascular wilt fusaria can penetrate host tissue directly, without requiring wounding of the host. The fungus then moves to the vascular tissue, entering the xylem vessel elements. It may later invade xylem parenchyma cells. It spreads throughout the plant by way of mycelia or microconidia. The fungus may eventually invade the pith, cambium, phloem, and cortex. Wilt symptoms consequently occur. *F. oxysporum* may be able to colonize other nonhost plants to survive, without causing disease. It can also persist in decaying plant debris for several years.

1.1.3 Previous research

Very little research has been conducted on *Fusarium* wilt of basil, because the disease has only recently become widespread, and basil is considered to be a minor crop. Vergovskii (1956) stated the high soil temperature at which basil seedlings are forced is responsible for the spread[1] of *Fusarium* wilt in nurseries in the former U.S.S.R.; therefore, he recommended that basil culture be restricted to soils that do not exceed 20C. Kvartskhava (1957) noted

[1] *Spread* was the word the translator of this article from the Russian language used: it is believed Vergovskii meant *severity* instead.

the pathogen invades the basil plants through injured roots, and that sources of infection are contaminated plant and weed residues, infected young transplants, and seed. *Fob* has been reported to be seedborne by many researchers (Kvartskhava, 1958; Sharma, 1976; Martini and Gullino, 1991; Keinath, 1993; Wick et al., 1993; Elmer et al., 1994). It has also recently been reported to be airborne: Gamliel et al. (1996) left opened agar plates in greenhouses containing diseased basil plants, and found that 56 of the 255 *F. oxysporum* isolates collected in this manner were pathogenic on basil. The disease has been reported in the field and in hydroponic culture as well (Wick and Haviland, 1992). The pathogen is reported to be specific to *Ocimum* species (Kvartskhava, 1957; Keinath, 1993, Keinath, 1994). It can colonize other plants in the Lamiaceae without causing disease (Keinath, 1994).

Dzidzariya (1961, 1964, 1968) was able to eliminate *F. oxysporum* from soil through the use of methyl bromide. The introduction of *Trichoderma* species into the soil rhizosphere also reduced *Fusarium* wilt severity and improved the green yield of basil. The biocontrol agent Mycostop (*Streptomyces griseoviridis*) and the fungicides mancozeb

(Manzate 200DF), iprodione (Rovral 4F) and benomyl (Benlate 50WP) were not consistently effective against *Fusarium* wilt of basil (Wick, unpublished data; Keinath, 1994). Research into breeding basil varieties resistant to *Fusarium* wilt is being carried out by Reuveni et al. (1995).

1.2 Objectives of the Study

A. Investigate the nature of the seedborne habit of *Fusarium oxysporum* f. sp. *basilicum* on basil (*Ocimum basilicum* L.).

B. Survey commercial basil seed to determine extent of problem.

C. Confirm the pathogenicity of *Fob* isolates found on commercial basil seed, through Koch's postulates and vegetative compatibility group (VCG) tests.

D. Determine the best method for eliminating the pathogen from basil seed.

   1. Benomyl/acetone treatments.
   2. Chitosan treatment.
   3. Mycostop treatments.
   4. Bleach (sodium hypochlorite) treatments.
   5. Hot water treatments.
   6. Bleach/hot water combination.

E. Perform grow-out tests in the greenhouse after seed treatments.

F. Determine the effectiveness of Mycostop as a soil drench in potting mix contaminated with *Fob*.

G. Survey composted potting mixes for their ability to suppress *Fob*.

CHAPTER 2

INVESTIGATING THE SEEDBORNE NATURE OF *FUSARIUM OXYSPORUM* F. SP. *BASILICUM* ON BASIL

2.1 Literature Review

A seed is a sexually derived structure of spermatophytes which germinates to produce new plants (Baker, 1972). The seed consists of an embryonic plant, stored food material, carried in the endosperm; and a protective seed coat (Agarwal and Sinclair, 1987). The embryo is comprised of the embryonic axis and the cotyledons. The axis combines the radicle (embryonic root), the hypocotyl to which the cotyledons are attached, and the shoot apex with the first true leaves (Bewley and Black, 1985). The endosperm results from fertilization in angiosperms (flowering plants). It is composed of cells rich in food reserves like starch, protein, or fats, which are utilized to feed the embryo. The seed coat develops from the outer cell layers of the ovule. The coat must possess great resistance to environmental extremes such as moisture and dessication (Laetsch, 1979).

Seeds can be both vehicles and victims of disease (Neergaard, 1979). It is therefore necessary to clarify terms such as "seedborne", "seedborne disease" and

"seedborne pathogen". If a seed "bears" a pathogen, the seed is a supporter and/or transmitter of the pathogen. A "seedborne pathogen", then, must be present in, on, or with the seed, but does not necessarily mean the seed is diseased. A "seedborne disease", on the other hand, is defined by Baker (1972) as a seed which exhibits symptoms. However, since the suffix "borne" relates to "carrying" pathogens rather than expressing disease symptoms, perhaps a better term for diseases only expressed on seeds is "seed disease" (Maude, 1996).

There are three types of seed/pathogen associations possible. Seedborne pathogens may be in, on, or associated with the seed. In other words, seed can either be infested (the pathogen is borne *on* the seed surface); infected (the pathogen is *inside* the seed, usually in a resting stage); or, the pathogen may be associated (*with*, but not on the seed).

Seed infestation, or contamination, refers to the passive relationship of a pathogen with seeds. Infective propagules of pathogens may adhere to seed surfaces during harvest or post harvest operations. Seed infection is the establishment of a pathogen within any part of a seed. This

may occur systemically through the vascular system, or directly through floral infection or penetration of the ovary wall, the seed coat, or other natural openings. All the seed parts can be infected. Seeds may be systemically infected from the flower or fruit stalk, through the seed stalk, to the seeds. Other fungi deposit spores on the stigma to germinate, consequently infecting the ovary and establishing in the seed. Other pathogens may directly penetrate the ovary wall or seed coat. They may subsequently enter the endosperm, cotyledons, or embryo, or remain in the seed coat. Other fungal pathogens may merely associate themselves with seed in the form of pathogenic propagules such as sclerotia, infected plant debris, or infested soil mixed with seed (Agarwal and Sinclair, 1987). For predictable seed transmission to occur, infection of a particular seed part is essential, depending on the host/pathogen association (Agarwal and Sinclair, 1987).

Transmission of a pathogen from the seed occurs either systemically or nonsystemically. Systemic seed transmission mainly occurs through infection of the embryo; and less frequently through infection of the endosperm, or the seed coat, or by seed coat contamination. Embryonic pathogens can

move systemically through the plant, following the growing point, and can express symptoms at different stages of plant growth. Nonsystemic seed transmission commonly results from seed coat infection, seed contamination or association. These seeds either fail to germinate, or infect young seedlings or cotyledons; perhaps leading to secondary spread of the pathogen (Agarwal and Sinclair, 1987).

Vascular fusaria are rarely exclusively seedborne; however, conidia or chlamydospores are frequently carried as surface contaminants on seed, or are associated through plant debris that remains with the seed after harvest. *F. oxysporum* forms have been found on the seeds of pea, tomato, bean, asparagus, sugarbeet, aster, plantago, and oil palm (Nelson, 1981). However, some fusaria may be carried internally as well as externally by seed. *F. oxysporum* f. sp. *mathiolae* (the causal agent of wilt of garden stock, *Matthiola incana*) infects seed by growing through the xylem, where it is internally transmitted (Baker, 1972). *F. oxysporum* f. sp. *cumini*, the causal agent of wilt of cumin, infects seeds not only superficially but internally as well. Microscopic studies performed by Singh et al. (1972)

revealed fungal hyphae lying intracellularly in the endosperm.

There are several methods of detecting the exact association between a fungus and a seed. Surface contamination and pathogen association can be examined by placing approximately 100 seeds in a flask with detergent (Tween) and water, and shaking the flask on a mechanical shaker for 5 to 10 minutes. The liquid is then centrifuged, and any resulting pellet is examined for fungal spores (Agarwal and Sinclair, 1987). A variant of this method was tried by Inglis (1980), who rinsed asparagus seeds contaminated with *Fusarium moniliforme* with tap water, and found contamination reduced from 2.3 to 0.33%. Surface sterilization of the seed exterior by simply soaking seed in 10% bleach (sodium hypochlorite) should confirm the seedborne relationship as well. Rudolph and Harrison (1945) delinted cotton seed with concentrated sulfuric acid, scraped off any bristle-like hairs with a scalpel, immersed the seed in 0.1% mercuric chloride for five minutes, and washed the seed in sterile water, and still found *Fusarium* contamination; therefore, they concluded the fungus was internally seedborne.

Basil seeds produce a layer of mucilage about 2 mm thick when soaked in water. The layer originates from inside the seed coat. The mucilage contains complex carbohydrates consisting primarily of polyuronides and galacturonides which chemically resemble pectins and hemicelluloses. Physically, mucilages are similar to the gums found in tree bark and stems of other plants (Copeland and McDonald, 1985). The function of the mucilage is either to aid in seed dissemination, or to facilitate more efficient water uptake by the seed (Mayer et al., 1982). No literature has been found which discusses the role of mucilage in fungal diseases. It is unknown as to whether the mucilage is able to harbor fungal spores.

2.2 Materials and Methods

2.2.1 Confirming the seedborne association

In this study, the method suggested by Agarwal and Sinclair was tried with basil seeds. One hundred basil seeds were placed in a 125 ml Erlenmeyer flask filled with 50 ml sterile water and two drops of Tween detergent. The flask was placed on a mechanical shaker for 10 minutes at 400 revolutions per minute (rpm). The liquid was drained from the flask into a centrifuge tube. The tube was

centrifuged at 4000 rpm for fifteen minutes. Upon not finding a pellet, a few drops of the liquid were placed on each of five plates of Komada media, which is selective for *Fusarium oxysporum* (Komada, 1975). The drops were spread upon the agar surface with a sterilized glass spreader. The plates were stored at 27°C for one week before they were checked for *Fusarium* growth. A drop of the liquid was also examined under the light microscope to see if any fungal spores could immediately be viewed. This experiment was replicated three times.

2.2.2 Detecting possible internal contamination of the seed embryo

Approximately 100 flower embryos were removed from artificially infected and noninfected basil plants. Twenty of the embryos were dissected with sterile forceps and needles under a dissecting microscope, placed on a microscope slide, stained with aniline blue, and viewed under a light microscope to look for mycelium. This method is a variant of a technique suggested by Singh et al. (1972), who soaked artificially inoculated cumin seeds with lactophenol for 24 hours before cutting transverse sections of seeds and staining them with cotton blue. Microscopic

examination of the sections found fungal hyphae in the endosperm.

The remaining flower embryos removed from both infested and noninfested basil plants were briefly surface sterilized in 10% Clorox™ (0.525% sodium hypochlorite), washed in sterile distilled water, and dried. The embryos were cut in half with a sterile razor blade and placed onto Komada media. The plates were incubated at 27°C and checked for *Fusarium* growth after one week.

2.2.3 Determining the mode of pathogen movement to the seed

To determine if *Fob* moves from the infected plant through the flower pedicels into the embryo, portions of the lower, middle, and upper stem, and the flower pedicels from inoculated basil plants exhibiting *Fusarium* wilt symptoms were removed aseptically from the plants. The polar end of each stem piece and pedicel was sliced off with a sterile razor blade before placing the pieces onto Komada/*Fusarium* agar plates. The plates were incubated at 27°C and inspected for *Fusarium* growth after one week.

Most basil plants infected with *Fob* die before producing seed. However, some plants artificially inoculated with *Fob* do not exhibit symptoms. It is

necessary to know if these asymptomatic plants still carry the fungus and transmit it to their progeny. One hundred and seventy seeds were collected by hand from asymptomatic plants manually infected with *Fob*, as were 300 seeds taken from inoculated, symptomatic plants. The seeds were plated directly onto Komada media to check for *Fob* contamination.

2.2.4 Examining the mucilaginous layer extruded from basil seed for *Fob*

At least twenty seeds were soaked in water until mucilage was exuded (about 15 minutes). The mucilage was then scraped off the seed with a sterilized needle, placed on a microscope slide, and stained with aniline blue for examination under the microscope.

2.3 Results and Discussion

2.3.1 Confirming the seedborne association

No pellet remained after centrifuging the water/detergent solution which had previously contained 100 basil seeds. The remaining liquid, after microscopic examination, also contained no conidia. There was no fungal growth upon the agar plates amended with the water/detergent solution. These results suggest but do not completely confirm internal contamination of the basil seed by *Fob*,

since the process of soaking and centrifuging may not have been capable of removing any *Fob* conidia adhering to the outer surface of the seeds. It may be surmised, though, that the contamination is not of the associative nature, because the conidia would have readily been removed from the seed area, as per the method tried by Inglis (1980).

2.3.2 Detecting possible internal contamination of the seed embryo

There was no mycelium seen in the embryos taken from noninfected basil. However, both the outer and internal portions of the embryos obtained from plants inoculated with *Fob* displayed copious mycelial growth. It was not confirmed as to whether the mycelia was that of *Fob*. Embryos plated onto Komada agar yielded no *Fusarium* growth.

One embryo taken from an asymptomatic plant artificially infected with *Fob* revealed a *Fusarium* isolate. The isolate was tested for pathogenicity upon basil, using the technique outlined in Chapter 4. It was shown through RAPD-PCR analysis to be *Fob*; however, the isolate was nonpathogenic upon basil; therefore, it can not properly be termed an *Fob* isolate.

2.3.3 Determining the mode of pathogen movement to the seed

There was no *Fusarium* growth emitting from each polar end of the pedicels plated onto Komada. On inoculated plants producing seed, there was *Fusarium* growth emitting from only the lower and mid-stem portions of the plant. Plants which wilted completely before bearing seed exhibited *Fusarium* growth throughout the entire plant.

None of the 470 seeds exhibited *Fob* contamination. A larger subsample of seeds from inoculated, asymptomatic basil plants should be retested; however, perhaps those few basil plants possessed at least some inherent resistance to *Fusarium* wilt, and passed this quality on to their progeny. If this is so, this finding agrees with studies performed by Reuveni et al. (1995), who identified symptomless basil plants grown in soil infested with *Fob*, and passed the inherent resistance to the subsequent generation of plants.

On the other hand, this finding may assert that the pathogen does not move through the plant to the seed, but instead relies on external seed contamination by *Fob* spores. The fungus may contaminate seeds during threshing, and may find a way to move into the seed through cracks or openings,

since it has been affirmed the fungus cannot be simply washed off the seed surface.

These results do not agree with what was reported by Gamliel et al. (1996), who found seeds extracted from diseased plants carried *Fob* and gave rise to diseased basil plants, which in turn produced a new generation of diseased seed.

2.3.4 Examining the mucilaginous layer extruded from basil seed for *Fob*

No conidia or hyphae were found in the mucilage scraped off the surface of basil seed. The removal of the mucilage layer through centrifugation should be the next step attempted. This may be difficult due to the strongly adherent nature of the mucilage to the seed, which made it difficult to scrape off the seed. Examining surface contamination through bleach (sodium hypochlorite) treatments will be discussed further in chapter 5, section 4.

CHAPTER 3
A SURVEY OF COMMERCIAL BASIL SEED FOR CONTAMINATION BY *FUSARIUM OXYSPORUM* F. SP. *BASILICUM*

3.1 Literature Review

It is desirable to ascertain the source and the severity of a disease outbreak in order to enact control measures. Commercial seed distribution is one of the most efficient ways to spread a disease to distant locations. Purveyors of seeds do not wish to have their reputations ruined by spreading a damaging disease with their product, and growers do not want to lose money through the devastation of their crops.

A routine method for screening seeds for disease must satisfy the following criteria, as stated by Neergaard (1977):

1. The test must give reliable information pertaining to field performance and quarantine regulations.

2. The results must be reproducible within statistical limits.

3. The time, labor, and equipment for carrying through a test must be kept with economic limits.

4. In tests requiring incubation, the results must be made available quickly.

The outcome of a seed health test presents three possibilities: the seed is suitable for sowing without treatment; the seed can be sown after treatment; or the seed is unsuitable for sowing (Neergaard, 1977). The seeds also may be inspected after a treatment used to eradicate the pathogen from the seed is applied.

There are several techniques utilized to screen seeds for disease.

1. Direct inspection either with the naked eye or with a microscope. This may include examination after application of a clearing and/or staining procedure.

2. Examination of suspensions obtained by the washings from the seed.

3. Examination of seeds after incubation on blotter paper or on agar media.

4. Tests based on serological or biochemical reactions, such as ELISA (enzyme-linked immunosorbent assay) or PCR (polymerase chain reaction) (Neergaard, 1977).

Direct inspection of basil seed embryos with a microscope, along with staining procedures, is discussed in Chapter 2, as is the examination of suspensions obtained by

washing the basil seed with sterile distilled water and detergent.

The blotter and agar plate methods are recommended by the International Seed Testing Association for routine examination of crop seeds for fungal infections. The identification of the fungus is based on its morphology as it develops on the seed surface on the blotter, or as colony characteristics on the agar medium (Agarwal and Sinclair, 1987). The principle behind the blotter method is to provide an environment conducive to fungal development, through conditions of high humidity and optimum light and temperature levels. A more reliable method for examining *F. oxysporum* occurrence on basil seed is through the agar plate method. The method is even more efficient if a medium selective to the target organism is utilized. Komada (1975) developed an agar medium which is particular to *Fusarium* species (Appendix A). It can be useful in distinguishing *F. oxysporum*, *F. solani*, *F. moniliforme*, and *F. roseum* by the color of the colonies on the agar.

It is necessary to screen as many commercial basil seed lots as possible to determine the extent of *Fob* contamination. Screening basil seed sources from throughout

the world for *Fob* may assist in locating the origin of *Fusarium* wilt of basil, and in the subsequent eradication of the disease. It is not known at this time how or from what country the disease originated; although the disease was first discovered in Russia in 1956, it was not reported elsewhere for nearly twenty years, until its discovery in Italy in 1975.

A DNA probe designed to detect *Fob* in basil seeds is being developed by Zheng Pan and Robert Wick, of the Department of Plant Pathology, University of Massachusetts. Since this research is still underway, the details will not be examined here.

3.2 Materials and Methods

Twenty-four commercial basil seed lots, obtained from at least five different countries, were utilized for this experiment. Between 300 to 500 seeds were tested from each lot.

For this study, the agar plate method utilizing Komada-*Fusarium* media was utilized. The agar media was prepared according to the recipe in Appendix A. Twenty-five basil seeds from each of the 24 commercial seed lots were placed directly onto the agar. At least 100 seeds were tested per

replication, from each seed lot. The plates were incubated in the dark at 27°C for 3 - 4 days before they were checked for *Fusarium* contamination. The experiment was replicated at least three times for every seed lot. The seed lots which were especially contaminated were replicated up to five times.

3.3 Results and Discussion

The contamination levels of each of the 24 seed lots are presented in Table 3.1. Eight of the seed lots exhibited no *Fusarium* contamination. Less than 5% of the seeds were contaminated in 13 of the lots. Only one lot demonstrated 25% *Fusarium* contamination. Overall, two-thirds (66.6%) of the 24 lots had some seedborne fusaria. This is an alarming number to any basil grower, and ensures that care must be taken when selecting a seed source.

Because of the lack of knowledge regarding the country of origin of most of these seed lots, it is unknown through this study where any tainted seeds tend to emanate from. It also cannot be stated that any one or several seed distributors are most responsible for the spread of the pathogen. Distributors gather their seed from many different locations and growers. It is suggested that

distributors concern themselves with discovering the sources of contaminated seed, and avoiding those sources if possible. Of course, this is a controversial subject; as no one enjoys taking responsibility for a seedborne disease epidemic. However, to stay the development of a serious epidemic, growers, distributors, and scientists must work together for the benefit of all.

Table 3.1 Testing of commercial seed sources for contamination with *Fusarium oxysporum* f. sp. *basilicum*.

Lot No.	Source No.[a]	Origin	% *Fo*[b]
3634-000	01	?	11
3635-000	01	U.S.A.	0
8295	02	?	0.75
None	03	Netherlands	0
1908	03	U.S.A.	0
ST159C	04	Italy	1
FLB91	04	Holland	1
HG J2409	04	U.S.A.	0
A8383K	05	?	4.5
49262-3D	06	?	1.3
9405	07	?	0.5
7331	08	?	4
7537	08	?	25
005-01-39	09	?	7
25-096-001	09	?	2
313-11-09	09	?	2
AW12	10	?	0

Continued, next page

Table 3.1 continued.

Lot No.	Source No.[a]	Origin	% *Fo*[b]
Z715	10	Germany	0
1290	11	?	0
1300	11	?	0
1330	11	?	3
742-131	11	?	2
4341	12	?	0.5
4724	12	?	1.2

[a]Commercial seed sources were assigned numbers to preserve their anonymity.
[b]Percentage of seeds yielding *Fusarium oxysporum* isolates after plating the seeds on Komada/*Fusarium* media.

CHAPTER 4
CONFIRMATION OF THE PATHOGENICITY OF SELECTED ISOLATES OF *FUSARIUM OXYSPORUM* F. SP. *BASILICUM*

4.1 Literature Review

4.1.1 Determining pathogenicity of strains of *F. oxysporum*

The classification of plant pathogenic fungi into hierarchically arranged groups (taxa) is an ongoing process among researchers. Many fungal characteristics have been utilized in past classifications; however, morphology has been the primary component of differentiation. This method of comparison is less useful for the plant pathologist, who needs to be more concerned with details regarding pathogenicity and host specificity. A physiological strain of a fungus which is morphologically and culturally indistinguishable from saprophytic strains, and which shows different abilities in parasitizing specific hosts is termed a *forma specialis* (Booth, 1971).

The term has utility in the classification of the genus *Fusarium*. An early mycological concept assumed all fusaria were particular to one host or host group. Consequently, every new host record was described as a new fungal species, leading to the naming of over 1000 species, varieties, and forms of fusaria (Booth, 1975). *Fusarium* is difficult to

characterize due to its wide variation in morphological and physiological characteristics (Snyder and Hansen, 1940). In their treatise characterizing the vascular wilt fusaria, Snyder and Hansen found the three sub-sections, 10 species, 18 varieties, and 12 forms comprising the *Elegans* section of the *Fusarium* genus were actually all similar in cultural appearance, morphology, and physiology, within well-defined limits. The only difference they found between the fungi was in regard to host pathogenicity. The Armstrongs, as stated in Chapter 1, subsequently devoted their professional lives to assembling the strains of the species into formae speciales and races according to their pathogenic effects on miscellaneous host plants.

4.1.2 Koch's postulates

The degree of pathogenicity a particular pathogen inflicts upon a host plant can be determined by inoculating the host with the specific isolate to be tested. Koch's postulates are implemented in plant pathology when a pathogenic agent isolated from a diseased plant is unknown or unreported on that plant host. The hypothesis that the isolated organism is the cause of the disease must be verified. The following steps are taken to determine this:

1. The pathogen must be found associated with the disease in all the diseased plants examined.

2. The pathogen must be isolated and grown in pure culture on nutrient media, its characteristics described, and its appearance and effects recorded.

3. The pathogen from pure culture must be inoculated on healthy plants of the same species or variety on which the disease appears, and it must produce the same disease on the inoculated plants.

4. The pathogen must be isolated in pure culture again, and its characteristics must be exactly like those observed in step 2 (Agrios, 1988).

4.1.3 VCG's and other tests

An advantage present and future researchers have, which the Armstrongs did not, is modern technology. Booth (1975) stated that any technique that would distinguish formae speciales or races in laboratories, without the tedium of repeated plant inoculations, would be of immense value to plant pathologists. There have been several techniques discovered within the past ten years which can show the genetic similarities and differences between strains of *F. oxysporum*. One technique is the use of vegetative

compatibility, established by documenting that vegetative heterokaryosis has occurred. Heterokaryosis is the capacity of haploid nuclei to form various associations within vegetative cells. Vegetative compatibility is the ability of the mycelia of two fungal strains to make contact, anastomose, and produce a viable heterokaryotic cell. Anastomosis involves fusion of hyphae, movement of one or more nuclei into one or the other of the fused cells, and the establishment of a compatible heterokaryotic state. Failure to establish heterokaryons may involve any one of those steps (Parmenter et al., 1963). Incompatible strains can exhibit areas of cell death. These events are governed by a set of genes named *vic* (for *v*egetative *inc*ompatibility) loci, which depict a means for discriminating self from non-self (Leslie, 1993). Compatible isolates form vegetative compatibility groups (VCG's). Since sexual recombination does not occur in *F. oxysporum*, isolates in one VCG are genetically isolated from isolates in other VCG's.

The most useful application of vegetative compatibility for plant pathologists is the use of VCG's as a diagnostic tool. The application depends on the hypothesis that strains in a forma specialis or race form one or a few VCG's

(Puhalla, 1984; Leslie, 1993), and nonpathogenic strains of the same species fall into different VCG's. However; there are not always obvious correlations between formae speciales and VCG's. Therefore, Koch's postulates should be utilized to test the correlation between VCG and pathogenicity.

In *F. oxysporum*, visual inspection of heterokaryosis is extremely difficult to confirm; therefore, mutants must be created to test for heterokaryosis. Mutants can be generated through exposure to ultraviolet radiation, or by selecting auxotrophic markers such as an amino acid, vitamin, or nucleic acid that the fungus could ordinarily synthesize for itself (Leslie, 1993). Puhalla (1985) utilized a method which demonstrated heterokaryon formation by generating mutants unable to reduce nitrate, called *nit* mutants. This was accomplished by growing isolates on a minimal medium (M1) containing two grams of nitrate ($NaNO_3$), (see Appendix B for recipe), then transferring them to potato dextrose agar (PDC) containing 15 g potassium chlorate ($KClO_3$). Most chlorate-resistant sectors were also unable to reduce nitrate. This was tested by transferring the chlorate-resistant sectors back to M1. If the isolate grew thinly but expansively (characteristic of nitrogen

starvation, unlike a wild type), it was unable to reduce nitrate, and thereby was termed a *nit* mutant. The *nit* mutants from different isolates were then paired on M1, and if a line of dense mycelial growth occurred at the point of anastomosis, they are considered complementary, and therefore in the same VCG.

Correll et al. (1987) modified this technique by introducing three mutant designations, all of which were observed through growth on different nitrogen sources. A *nit*1 mutant was mutated at the nitrate reductase structural locus through its growth on PDC, and grew thinly on M1 but as a wild-type on M2 (minimal media amended with 0.5 g/L nitrite ($NaNO_2$) instead of nitrate), and M3 (minimal media amended with 0.2 g/L hypoxanthine instead of nitrate). *Nit*3 mutants mutated at a pathway-specific regulatory locus for induction of nitrate and nitrite reductases, and grew as a wild-type only on M3 media; and *nit*M mutants mutated at the molybdenum cofactor loci, and grow as wild types only on M2 media. *Nit*M strains were used as testers, because they most reliably complemented the other two strain types, which do not always complement each other.

VCG's in *F. oxysporum* have been compared in other formae speciales, races within a forma specialis, and formae speciales/races within a geographic locality (Elmer and Stephens, 1989). There have not always been correlations between VCG's and formae speciales/races. Elmer et al. (1994) compared 45 isolates of *Fob* taken from diseased basil plants and infested seeds from around the U.S. and from Italy. They found that 44 of the 45 isolates were vegetatively compatible with each other and also exhibited wilt symptoms when inoculated onto basil plants. The odd isolate, NY63, did not cause wilt on basil and was not vegetatively compatible with the other strains. This finding supported Puhalla's hypothesis that a forma specialis such as *Fob* can belong to one VCG, and nonpathogenic strains will not form heterokaryons with this group.

Another process recently adopted by Pan and Wick (1995) to differentiate *Fob* from other *Fusarium* species is through polymerase chain reaction (PCR) technology. A 0.7 kilobase fragment unique to *Fob* was collected, cloned into a plasmid vector, and labeled with nonradioactive digoxigenin-dUTP.

The probe specifically bound to amplified DNA from *Fob*, but not to the other *Fusarium* species tested.

In this study, strains of *Fob* isolated from seed and diseased plants were inoculated onto healthy basil plants in order to perform Koch's postulates. Some of the isolates were also subjected to VCG testing and PCR/probe analysis.

4.2 Materials and Methods

4.2.1 Koch's postulates

Isolates used in the study were taken from basil seed and diseased plants. Thirty-three isolates were tested.

Basil seeds from a seed lot thought to be free of *Fob* were hot water treated at 58°C for 20 minutes, then planted in a seedling tray containing 200 1.5 x 1.5 cm blocks filled with potting soil. All trays were placed on heating pads and placed in a mist room in order for the seeds to receive adequate moisture. After two to three weeks of growth, the plants were transferred to 10 cm pots containing potting soil.

Twenty-nine of the 33 *Fob* isolates were taken from a single spore culture placed in sterilized peat and stored at 3°C. The other four isolates were stored on potato dextrose

agar (PDA) slants. All isolates were transferred to and grown on minimal media (M1) for 7 - 10 days.

When the basil plants were four weeks old, the roots of each plant were stabbed with a plastic stick, and a spore suspension of the appropriate isolate was poured into the soil surrounding the root area. Inoculum was prepared by pouring sterile distilled water onto the petri dish containing the isolate, and scraping the colony with a sterilized glass spreader. Three basil plants were treated with each *Fob* isolate. Control plants were inoculated with sterile distilled water only. One isolate, Misc28, is known to be pathogenic on basil, and was used as a "positive" control. The isolate found by Elmer et al. (1994), NY63, to be nonpathogenic on basil was also tested. The plants were checked twice a week for wilt symptoms. Height of the plants was measured once a week. Plants demonstrating wilt symptoms were harvested, and the stems were cut off with a sterile razor blade and plated onto Komada/*Fusarium* media to confirm *Fusarium* was the cause of the wilt.

4.2.2 VCG testing

Six of the isolates utilized in direct pathogenicity testing (Koch's postulates) also underwent VCG testing. The

isolates stored in refrigeration in test tubes containing sterilized peat were then grown on M1. Single spore isolates were generated if they were not readily available, onto a small agar plate of M1.

Procedures for selecting, characterizing, and pairing the *nit* mutants in heterokaryon tests were described by Correll et al. (1987) and Elmer et al. (1994). After 3 - 4 days of growth on M1, four colonized agar plugs were transferred to plates of PDA supplemented with 15 grams of $KClO_3$ (PDC medium). After 7 - 14 days, hyphal tips of fast growing, chlorate resistant sectors were transferred to M1. Colonies exhibiting thin, expansive growth with no aerial mycelium were transferred to M2 and M3 to assign them to phenotypic classes. Any colonies exhibiting wild-type growth on M1 were discarded. The isolate demonstrating a *nit*M mutation (in this case, the NY63 and the D3 isolates were separately utilized) was placed in the center of an M1 plate. Isolates displaying a *nit*1 mutation, which is more common, were placed around the *nit*M isolate, about 1 - 3 cm apart from one another, in a "daisy " pattern. The plate was checked for heterokaryosis 7 - 14 days later. If dense, aerial growth developed where mycelia of the *nit* colonies

come in contact, a heterokaryon was assumed to have formed, and was considered evidence of heterokaryosis and vegetative compatibility.

The RAPD analysis of *Fob* and other *Fusarium* species used as comparisons was carried out by Zheng Pan. The isolates analyzed were: A1, A3, C4, C6, D3, L1 (from diseased seed), EM1 (from a basil seed embryo), Misc59 (an isolate obtained from the American Type Culture Collection by W.H. Elmer), and BSR1 (an isolate from a diseased basil plant).

4.3 Results and Discussion

4.3.1 Koch's postulates

The results of the pathogenicity tests are listed in Table 4.1. Twenty-five of the 29 isolates taken from the seed lot displaying 25% *Fob* contamination were pathogenic on basil. Only four strains were nonpathogenic on basil, establishing that these isolates were either saprophytes or another forma specialis of *F. oxysporum*.

The isolates found on a seed and an embryonic seed taken directly from inoculated but asymptomatic basil plants (see Chapter 2) were found to be nonpathogenic on basil.

4.3.2 VCG testing

Only isolate Misc59 formed a heterokaryon with both *nit*M strains, NY63 and D3. A *nit*1 mutant of D3 formed a heterokaryon with NY63. All three are nonpathogenic upon basil. The other isolates which have not induced wilt symptoms upon basil (C4 and C6) did not form a heterokaryon with the NY63 isolate. Isolate A3, a pathogenic *Fob* isolate, did not form a heterokaryon with either NY63 or D3. All six isolates were found to be *Fob*, according to RAPD analysis. This suggests there may be two separate VCG's of nonpathogenic *Fob*.

It appears even though RAPD analysis is a far quicker procedure than a VCG test, RAPD analyses are unable to distinguish between pathogenic and nonpathogenic *Fob* isolates at the present time. The evidence presented both here and by Elmer et al. (1994) suggests pathogenic and nonpathogenic strains belong to different VCG's. VCG testing is more rapid than pathogenicity tests; therefore, VCG testing appears to be the best method to distinguish pathogenic strains of *Fob*.

Table 4.1 Results of pathogenicity tests involving isolates of *Fusarium oxysporum* f. sp. *basilicum*.

Isolate	Origin	Pathogenic on basil	VC with NY63M	RAPD test
A1	L2S8[a]	No	----	Not *Fob*
A2	L2S8	Yes	----	----
A3	L2S8	Yes	No	*Fob*
B1	L2S8	Yes	----	----
B2	L2S8	Yes	----	----
C1	L2S8	Yes	----	----
C3	L2S8	Yes	----	----
C4	L2S8	No	No	*Fob*
C6	L2S8	No	No	*Fob*
D1	L2S8	Yes	----	----
D3	L2S8	No	Yes	*Fob*
E1	L2S8	Yes	----	----
F1	L2S8	Yes	----	----
F2	L2S8	Yes	----	----
F4	L2S8	Yes	----	----
F5	L2S8	Yes	----	----
F6	L2S8	Yes	----	----

Continued, next page

Table 4.1 continued.

Isolate	Origin	Pathogenic on basil	VC with NY63M	RAPD test
G2	L2S8	Yes	----	----
H1	L2S8	Yes	----	----
H6	L2S8	Yes	----	----
I2	L2S8	Yes	----	----
I3	L2S8	Yes	----	----
J3	L2S8	Yes	----	----
J4	L2S8	Yes	----	----
J5	L2S8	Yes	----	----
K1	L2S8	Yes	----	----
K2	L2S8	Yes	----	----
K3	L2S8	Yes	----	----
K5	L2S8	Yes	----	----
Misc 59	ATCC[b]	No	Yes	*Fob*
Emb.1	UM[c]	No	----	*Fob*
L1	UM	No	----	Not *Fob*
Misc 28	UM	Yes	No	*Fob*

[a]See Table 3.1; isolate is from seed lot 2, source 8.
[b]Isolate obtained from American Type Culture Collection.
[c]Isolate obtained from plants of seed grown at the University of Massachusetts.

CHAPTER 5
ELIMINATING *FUSARIUM OXYSPORUM* F. SP. *BASILICUM* FROM BASIL SEED

5.1 Benomyl/Acetone Treatments

5.1.1 Literature review

The use of fungicides as seed treatments is the most widely followed disease control practice used in all crops (Agarwal and Sinclair, 1987). Fungicides can be used to either disinfect (eliminate pathogens established within the seed or seed coat) or to disinfest (eliminate pathogens outside the seed or on the seed surface). Seed treatment methods include: dry application of fungicide, seed dips, fumigation, slurry treatment, or mist treatment.

Systemic fungicides are most useful for eradicating internally seedborne pathogens. Examples of systemic fungicides used on seedborne pathogens include carboxin, for the control of covered smut of oats caused by *Ustilago hordei*; and benomyl, for the control of gray mold of onion caused by *Botrytis allii* (Agarwal and Sinclair, 1987).

A problem is the incorporation of the chemical fungicide into a seed. This can be overcome through the use of a volatile organic solvent as a fungicide carrier. The carrier should not cause any damage to the seed, and should

evaporate after carrying the chemical into the seed, while leaving the fungicide inside. Carriers include DCM (dichloromethane), PEG (polyethylene glycol), and acetone.

Acetone was found by Milborrow (1963) to transport compounds into seeds without compromising germination potential. Some seed types could be soaked for periods of up to six months and still germinate readily, provided the acetone was washed off before the seeds were soaked in water.

The permeation and translocation of carboxin and ethazol applied in acetone for treatment of cotton and soybean seed was investigated by O'Neill et al. (1979). They found that seeds took up and retained more fungicides in the seed coat and embryonic tissues when immersed in solvent solutions such as acetone, than when treated directly by tumbling the seeds with the chemicals in glass jars, without a carrier. The fungicide and the solvent reached maximum concentration under the seed coat in approximately 15 minutes.

The systemic fungicide benomyl (methyl 1-[butylcarbamoyl]-2-benzimidazolecarbamate) is in the benzimidazole group of systemics, which were introduced in

1968. Benomyl has the widest spectrum of fungitoxic activity of the newer systemics (Ware, 1983), and is effective at relatively low doses. Most ascomycetes, and some basidiomycetes and deuteromycetes are sensitive to benomyl. The effects of benomyl on systemic pathogens such as *Verticillium*, *Fusarium*, and *Ceratocystis* were promising under controlled conditions, but distribution in soil and host is frequently insufficient for desired results on some hosts (Delp, 1987). Benomyl can be used in foliar applications, dipping of fruit or roots, soil application, and seed treatment (Ware, 1983).

The latter use was tried by Damicone et al. (1981) on asparagus seeds infested with *Fusarium moniliforme* (now called *F. proliferatum*) and *F. oxysporum*. Three benomyl quantities (6,250, 12,500, and 25,000 ppm) were suspended in acetone for the experiment. Seeds were soaked in the solutions for 24 hours. After treatment, the seeds were repeatedly washed in sterile, distilled water to remove benomyl residues, left to soak for 24 hours in sterile distilled water, and placed on potato carrot agar acidified with lactic acid (PCAL). All three benomyl/acetone treatments eliminated *F. moniliforme* from the asparagus seed

with only slight germination loss; however, *F. oxysporum* was only eliminated at the treatment with the highest benomyl content of 25,000 ppm.

Daniels (1983) attempted two treatments of 6,250 ppm and 25,000 ppm benomyl in acetone to eliminate *F. moniliforme* from corn seed. Seed infestation was reduced by 100% by both treatments; however, seedling infestation was only decreased by 25% and 85% by the 6,250 ppm and the 25,000 ppm treatments, respectively. Germination was reduced by 30% and 35%, respectively.

5.1.2 Materials and methods

Two benomyl/acetone treatments of 12,000 ppm and 25,000 ppm benomyl were attempted according to the procedure utilized by Damicone et al. (1981). 1.2 grams and 2.5 grams of Benlate (benomyl) were added each to 100 mls of acetone in two 250 ml Erlenmeyer flasks, under a fume hood, to make a 12,000 ppm and a 25,000 ppm treatment, respectively. A stir bar was also added to each flask, and the flasks were stirred on stirring machines for approximately ten minutes so the benomyl would dissolve. Two hundred basil seeds from a seed lot with a 25% incidence of *Fo* contamination were added to each mixture. The flasks were placed on a shaker

for 24 hours at 150 rpm at 27°C. After the 24 hours, the seeds were rinsed twice with acetone, then rinsed ten times with sterile, distilled water. They were dried on sterile filter paper under a transfer hood. Half the seeds from each treatment were plated onto Komada medium; and the other half onto moistened sterile filter paper in glass petri dishes. All plates were incubated at 27°C for at least two weeks. Since none of the benomyl-treated seeds germinated, it was thought perhaps acetone may have an adverse effect on basil seeds. A preliminary seed treatment with acetone alone was attempted to discover this. Five soaking times were undertaken: 30 minutes, one hour, four hours, eight hours, and 12 hours. For each soaking time, one hundred basil seeds were soaked in 25 mls of 99% acetone. After the soaking period was concluded, the seeds were rinsed several times with sterile, distilled water, then soaked for 24 hours in sterile distilled water before plating the seeds onto Komada/*Fusarium* media. The plates were incubated for 4 - 7 days and checked repeatedly for *Fusarium* growth.

5.1.3 Results and discussion

The seeds soaked in 99% acetone alone showed a notable reduction in seed germination, regardless of the length of

treatment. None of the seeds soaked for 12 hours survived the treatment. Only 25% of the seeds germinated after a 30 minute or a 1 hour soak in the acetone. Apparently basil seeds are sensitive to acetone, unlike soybean and other types of seeds. This could be due to the small size of the basil seeds, or perhaps the seed provides no means for escape of the acetone through evaporation. In any case, the benomyl/acetone treatments were deemed inappropriate for basil seeds.

5.2 Chitosan Treatments

5.2.1 Literature review

Chitin, a common constituent of fungal cell walls, has been utilized as a chemical control method against soilborne fungi, especially *Fusarium* (Mitchell and Alexander, 1961). Chitosan, the deacetylated derivative of chitin, is also a component of the cell walls of some fungi, although to a lesser extent than chitin (Allan and Hadwiger, 1979). *Fusarium oxysporum* and *F. solani* are known to be sensitive to chitosan, due to chitosan's ability to inhibit spore germination and growth. The mechanism of inhibition is not completely known, but Hadwiger et al. (1985) have indicated that the synthesis and accumulation of fungal RNA are

obstructed by chitosan. The plant host is also induced to respond to pathogen invasions by accumulating β-1,3 glucans, phenols, and ligninlike compounds (Benhamou et al., 1994).

Chitosan is a polycationic molecule; therefore, it readily coats seed surfaces with an adhering glossy coat (Hadwiger et al., 1985). Benhamou et al. (1994) were able to significantly decrease incidence of *Fusarium* crown and root rot of tomato, caused by *F. oxysporum* f. sp. *radicis-lycopersici*, through a seed treatment of 1 mg/ml solution of chitosan.

5.2.2 Materials and methods

The chitosan solution was prepared according to Benhamou et al. (1994). Approximately five grams of crabshell chitosan (Sigma, Lot 34H1303, St. Louis, MO) was ground to a powder in a Wiley mill to pass through a 60 mesh sieve. The chitosan was then placed in a 500 mesh sieve and washed repeatedly in distilled water. The chitosan was centrifuged in distilled water at low speed (1000 RPM for five minutes), and the resulting pellets were air-dried for three days. After drying, approximately two grams of the chitosan were placed in centrifuge tubes containing 0.25 N hydrochloric acid. The tubes were centrifuged for 10

minutes at 1000 RPM to remove insoluble material. The chitosan was solubilized by the addition of the HCl; therefore, any insoluble material was removed from the centrifuge tube and the liquid was saved. A solution of 2.5 N sodium hydroxide was added dropwise while stirring to the liquid until the chitosan precipitated. The material was pelleted by centrifugation at 2500 RPM for 15 minutes, and washed several times with deionized water to remove salts. Every 50 mls of the rinse water was checked for salts using an electrical conductivity meter (Yellow Springs International, Model 32), until the meter read zero. To prepare the seed coating mixture, one gram of the chitosan mixture was dissolved in 0.25 N hydrochloric acid under continuous stirring, and the pH was adjusted to 5.6 using 1 N sodium hydroxide. The solution was diluted in sterile distilled water containing 0.01% (v/v) Tween 80 to obtain a final chitosan concentration of 1 mg/ml. Two hundred seeds known to exhibit 25% *Fo* contamination were soaked in 50 mls of the solution for 15 minutes. After the seeds were removed, half were placed directly onto Komada/*Fusarium* medium, and the other half were placed onto filter papers inside sterile petri dishes. Both sets of plates were

incubated at 27°C for 3 days, then checked daily for seed germination and *Fusarium* contamination. The experiment was carried out twice.

5.2.3 Results and discussion

Ten days after treatment, 94% of the seeds had germinated; however, at least one-quarter of those manifested leaf, stem, and/or root browning. Eventually the seedlings wilted and died. Seeds cultured on the Komada plates displayed an average of 8.5% *Fusarium* contamination (data not shown).

The chitosan solution utilized to coat the basil seeds is a complicated and time-consuming mixture to produce. Even if the treatment was completely successful in eliminating *Fob* from seed, it would not be simple for home gardeners and even for large producers of basil to implement. This method could be fine-tuned by adding more chitosan to the mixture, changing the soaking time period, or modifying the preparation procedures; however, it was decided to instead try the other methods mentioned later in this chapter, to see if they would be quicker, easier, and cleaner than a chitosan treatment.

5.3 Mycostop Treatments

5.3.1 Literature review

The use of biological control methods to manage plant diseases began centuries ago, but few of these processes have been utilized for seed treatments. Biological control is accomplished through manipulation of the environment, host, or antagonists. Antagonistic microorganisms interact with pathogens through competition, antibiosis, parasitism, cross protection, or induced resistance (Marois, 1990). Antagonists commonly applied to seeds include *Bacillus subtilis*, *Chaetomium* spp., *Penicillium oxalicum*, and *Trichoderma* spp. Their application to seed decreases seedborne fungi and results in vigorous seedlings. However, the use of some biological control agents has not been proven to be practical under field conditions, due to variables such as failure of antagonist carriers, environmental effects, and the difficulty of storing treated seed (Agarwal and Sinclair, 1987).

Mycostop® is a biological fungicide based on the soil bacterium, *Streptomyces griseoviridis*. The strain, isolated from *Sphagnum* peat, is normally present in nature and has not been genetically modified. The microbe deprives

pathogenic fungi of living space and nourishment by colonizing plant roots in advance of the fungi; and secretes various enzymes and metabolites which inhibit pathogen growth. *Fusarium* is a primary target of Mycostop.

Mycostop is used for the control of seed rot, root and stem rot, and wilt caused by various pathogenic fungi, of container grown ornamentals, herbs, and vegetables. Mycostop has been tested as a seed treatment on various ornamentals, leaf vegetables, root crops, and herbs. It can be mixed with seed in a planter box, or the seeds can be coated by mixing them with Mycostop in a small container. The use of Mycostop as a transplant dip and a soil drench will be discussed in Chapter 7.

5.3.2 Materials and methods

A preliminary seed treatment was performed by placing 200 basil seeds known to have an incidence of 25% *Fo* contamination, in a small glass jar along with a one gram packet of Mycostop®. The jar was shaken until the seeds became coated with the dry powder. Half the seeds were plated onto Komada/*Fusarium* medium; and the other half were placed into sterile petri dishes containing filter paper. Both sets of plates were incubated at 27°C for two days, and

were subsequently checked for *Fusarium* contamination and seed germination, respectively. This experiment was only performed once; because Mycostop works best as a root colonizer, it was concluded that greenhouse grow-out tests of plants treated with Mycostop and inoculated with *Fob* might give a better indication of treatment results. A combination of seed treatments and soil drenches was also undertaken. Those experiments are discussed further in Chapters 6 and 7, respectively.

5.3.3 Results and discussion

Seventy-seven percent of the seeds germinated, although 15 of those seeds exhibited browning of the leaves, stem, and/or roots. Ten percent of the seeds plated on Komada medium were contaminated with *Fusarium* (data not shown), which is a notable reduction considering the initially high level of contamination. However, 10% contamination is unacceptably high for purposes stated earlier.

5.4 Bleach (Sodium Hypochlorite) Treatments

5.4.1 Literature review

Chlorine bleach (sodium hypochlorite) is often utilized as a surface disinfestant for seeds and other plant parts. It is recommended for eradicating fusaria from seed surfaces

(Damicone et al., 1981); however, if the pathogen resides partially or totally within the seed, bleach treatments will not be effective. For example, bleach treatments were able to reduce but not completely exclude *Fusarium moniliforme* and *F. oxysporum* from asparagus seed (Damicone et al., 1981; Elmer and Stephens, 1988) or *F. moniliforme* from corn seed (Daniels, 1983). In tandem with a treatment which is able to kill an internal seed pathogen, such as a systemic fungicide/acetone treatment or a hot water treatment, bleach treatments work quite effectively (Elmer and Stephens, 1988).

Several different concentrations and soaking time periods have been utilized by various researchers. Damicone et al. (1981) soaked asparagus seeds in 10% Clorox™ (0.525% NaOCl) for ten minutes. The treatment reduced *F. oxysporum* contamination by nearly two-thirds, without affecting germination rates. Daniels (1983), attempting the same treatment on corn seeds, found a 75% reduction in seed infestation and a 65% reduction in seedling damage caused by *F. moniliforme*. Elmer and Stephens (1988), on the other hand, were able to reduce fungal contamination from 73% to 2%, with a 30 minute soak in 20% Clorox™ with 3 drops of

Tween 20. Chun and Schneider (1994) stated that buffering a 2.6% NaOCl solution to pH 7, from pH 11.6, significantly reduced rice seed contamination by fungi and bacteria.

5.4.2 Materials and methods

Four treatments, utilizing changes in NaOCl concentration, soaking times, and pH were attempted. The treatments were:

a. 20 minute soak in 10% Clorox™ (0.525% NaOCl);

b. 1 hour soak in 10% Clorox;

c. 2 hour soak in 10% Clorox; and

d. 20 minute soak in 10% Clorox buffered to pH 7 with 1 N HCl.

Two hundred seeds known to exhibit 25% *Fo* contamination were utilized per treatment. The seeds were soaked in their respective solutions for the corresponding time period. After treatment, the seeds were rinsed in sterile distilled water. Half the seeds from each treatment were plated onto Komada medium and the other half were plated onto filter papers inserted into sterilized glass petri dishes. A control (untreated seed) treatment was also included. The plates were incubated at 27°C for three days, after which

they were checked daily for seed germination and *Fusarium* contamination.

5.4.3 Results and discussion

The results of the bleach treatments are presented in Table 5.1. No bleach treatment was able to completely eliminate *Fusarium;* however, the reduction of contamination was always significant. Germination rates were never significantly affected by any of the bleach treatments. Nevertheless, a bleach treatment alone is not a viable answer to the control of *Fob* on basil seeds; however, in tandem with another treatment method, it may be a successful assistant.

The results also suggest that *Fob* is mostly a surface contaminant of basil seed, but some basil seed harbor internal infections as well.

5.5 Hot Water Treatments

5.5.1 Literature review

The control of plant pathogens through heat treatment has been practiced for over 100 years. Applied through water, air, or vapor, this method consists of heat treatment of seeds or plant parts at temperature and time regimes which kill the selected pathogen, without significant injury

to the host. The benefits and disadvantages of hot water treatments on seeds or other plant material were summarized by Baker (1962). The consequences of extreme heat on living organisms include enzyme denaturation, the melting of fats, destruction of hormones, tissue asphyxiation, and depletion of food reserves. For a hot water treatment to be successful, the plant pathogen must be more sensitive to heat than its host plant. This is possible due to the nature of the enzyme systems of parasitic microorganisms, which are generally more specialized than those of saprophytes. The thermal death point of the host plant must be considerably higher than that of the parasite. The heat tolerance of seed depends on the seed's moisture content, the age and vigor of the seed, the size of the seed being treated, the physical condition of the seed, and the varietal susceptibility of the cultivar. Problems with hot water treatments include asphyxiation of host tissues, premature germination, or -- a predicament with basil seeds -- the exudation of mucilaginous material which sticks the seed together upon drying. It is also unknown if seeds which exude mucilage are able to be soaked in water and still be

viable for planting at a later date, or if planting immediately after treatment is required.

The recommended heating interval for seeds smaller than 0.6 cm is 30 minutes. Presoaking the seed in cold water for four to twelve hours is practiced to eliminate air between the seed coat and the internal portion of the seed, because water is a better heat conductor than air. The pathogen may also be stimulated to grow during this presoak, rendering it more susceptible to the heat treatments. It is best to use water/tissue ratios exceeding 100:1 by weight, for treatments of 30 minutes or less (Baker, 1962).

5.5.2 Materials and methods

The thermal death point temperatures for both basil seed and *Fob* were determined by adding fifty seeds known to be contaminated with *Fob* to a glass test tube containing 4 mls of distilled water at room temperature. At least two test tubes were utilized per temperature treatment. The seeds were presoaked for at least four hours in the water before heating. The test tubes were placed in a preheated test tube heater (Barnstead/Thermolyne Modular Dri-Bath, Model No. DB16525) and heated for 20 minutes at the desired temperature. The test temperatures were 50°C (122°F) and

60°C (140°F). A room temperature treatment was utilized as a control. After the soak, the test tube contents were poured into a sieve to drain out the water. The seeds in the sieve were rinsed with cold, distilled water, then dried on sterile filter paper under the hood. Germination was examined by placing seeds in a glass petri dish (100 x 15 mm) with a moistened sterile filter paper inside (Fisherbrand filter paper, diameter 9.0 cm). The plates were checked after 3 - 4 days for seed germination.

Germination was reduced by more than 50% at 60°C. The germination potential at 50°C and at room temperature were equal; both exhibited about 97% germination. Based on these results, tests were carried out at 54°C (129.2°F); 56°C (132.8°F); 58°C (136.4°F); and 60°C (140.0°F), along with a room temperature (unheated) control. The treatments were carried out as stated above; except to check *Fusarium* contamination, half of the treated seeds were plated onto Komada medium (25 basil seeds per 100 x 15 mm petri dish). The plates were inspected for *Fusarium* growth after 3 - 4 days. The experiment was performed four times.

A test was also carried out to discover if hot water treatment of basil seed would result in a loss of

germination after 24 hours or one week or storage. Two hundred basil seeds were soaked in distilled water for four hours. They were then dried under the sterile hood for one hour, on sterilized filter paper. The seeds were incubated at room temperature under the sterile hood, half for 24 hours and half for one week. Afterward, the seeds were removed from the paper with a forceps and plated onto wetted sterile filter paper inside glass petri dishes. The dishes were incubated at 27°C for at least one week.

5.5.3 Results and discussion

There were no significant differences in germination between the room temperature control and the 54°C or 56°C treatments (Table 5.2). Germination was decreased by 20.25% by the 58°C treatment, and by 42.5% by the 60°C treatment. Variation between replications ranged from 17% to 65% under the 60°C treatment.

The hot water treatments significantly reduced *Fob* contamination (Table 5.3); however, the fungus was not completely eliminated from the seed until the water temperature reached 60°C. Since this lot of basil seed also demonstrated high rates of heat kill at this temperature, this indicates that the thermal death point of basil seed is

very close to the thermal death point of *Fob*. *Fusarium oxysporum* is said to be one of the most heat resistant fungi (Hoitink and Fahy, 1986). It is also one of the more long-lived soilborne fungi, making *Fusarium oxysporum* one of the more difficult plant pathogens to control.

The mucilaginous later secreted from the basil seeds dried quickly on the filter paper. When the seeds were removed from the filter paper after 24 hours, the dried mucilage caused them to stick to the paper, creating some difficulty in removing the seeds. At least fourteen seeds shattered during attempts at removal with the forceps. Out of 86 seeds removed from the filter paper, 64 germinated in the glass petri dishes after a 24 hour drying period. Several of the seeds exhibited contamination with *Fusarium* and *Aspergillus*, however. Seventy-six of 100 seeds left to dry for a week germinated. It can be concluded that germination was not impaired by drying the seeds; however, a more suitable substrate for drying and perhaps of seed removal needs to be devised.

5.6 Bleach/Hot Water Combination

5.6.1 Literature review

Since sodium hypochlorite (bleach) is utilized as a surface sterilizant, and hot water treatments are successful against internally seedborne pathogens, a combination of the two practices should achieve what each treatment separately cannot accomplish against a seedborne pathogen which may live both inside and outside the seed.

No studies were found which utilized both of these strategies together; however, several researchers have utilized chemical treatments in tandem with a hot water treatment (Grondeau and Samson, 1994). Elmer and Stephens (1988) followed a 30 minute, 20% bleach soak with a 24 hour benomyl (25,000 ppm)/acetone treatment on asparagus seeds, with success; fungal contamination was eliminated completely, with no reduction in germination. Reversing the treatment order (that is, soaking in benomyl/acetone prior to the bleach treatment) did not alter the results.

5.6.2 Materials and methods

One hundred basil seeds with a 25% incidence of *Fo* contamination were placed into a 50 ml glass beaker. A solution of 10% Clorox™ (0.525% NaOCl) was added to the

beaker, and the seeds were left to soak for ten minutes. The seeds were rinsed with sterile, distilled water several times before placing them into glass test tubes containing 4 mls of distilled water. The tubes were placed in a preheated test tube heater (see section 5.5, Materials and Methods) for 20 minutes, at a temperature of 55°C. After heating, the seeds were removed, dried under the sterile hood on pieces of sterile filter paper, and arranged onto petri dishes containing Komada/*Fusarium* agar. A control was prepared with one hundred nontreated seeds for comparison. The plates were incubated at 27°C and checked daily for germination and *Fusarium* contamination. The experiment was replicated three times.

5.6.3 Results and discussion

No *Fusarium* was cultured from any of the three hundred treated seeds, as compared to the control, which exhibited 20% *Fusarium* contamination. Seed germination decreased after the bleach/hot water treatment, to 68%, as compared to 90% germination for the control.

The bleach/hot water combination was the most successful of all the seed treatments attempted in this study. It is likely that since neither treatment alone was

able to completely eliminate *Fob* from seed, that most infected basil seed harbors the fungus both internally and externally; although as indicated by the significant decontamination of the seed by the bleach treatment alone, *Fob* is mainly an external contaminant of basil seed. The amount of internal infection, although modest, warrants the implementation of a hot water treatment as well.

incubated at 27°C for 3 days, then checked daily for seed germination and *Fusarium* contamination. The experiment was carried out twice.

5.2.3 Results and discussion

Ten days after treatment, 94% of the seeds had germinated; however, at least one-quarter of those manifested leaf, stem, and/or root browning. Eventually the seedlings wilted and died. Seeds cultured on the Komada plates displayed an average of 8.5% *Fusarium* contamination (data not shown).

The chitosan solution utilized to coat the basil seeds is a complicated and time-consuming mixture to produce. Even if the treatment was completely successful in eliminating *Fob* from seed, it would not be simple for home gardeners and even for large producers of basil to implement. This method could be fine-tuned by adding more chitosan to the mixture, changing the soaking time period, or modifying the preparation procedures; however, it was decided to instead try the other methods mentioned later in this chapter, to see if they would be quicker, easier, and cleaner than a chitosan treatment.

5.3 Mycostop Treatments

5.3.1 Literature review

The use of biological control methods to manage plant diseases began centuries ago, but few of these processes have been utilized for seed treatments. Biological control is accomplished through manipulation of the environment, host, or antagonists. Antagonistic microorganisms interact with pathogens through competition, antibiosis, parasitism, cross protection, or induced resistance (Marois, 1990). Antagonists commonly applied to seeds include *Bacillus subtilis*, *Chaetomium* spp., *Penicillium oxalicum*, and *Trichoderma* spp. Their application to seed decreases seedborne fungi and results in vigorous seedlings. However, the use of some biological control agents has not been proven to be practical under field conditions, due to variables such as failure of antagonist carriers, environmental effects, and the difficulty of storing treated seed (Agarwal and Sinclair, 1987).

Mycostop® is a biological fungicide based on the soil bacterium, *Streptomyces griseoviridis*. The strain, isolated from *Sphagnum* peat, is normally present in nature and has not been genetically modified. The microbe deprives pathogenic fungi of living space and nourishment by

colonizing plant roots in advance of the fungi; and secretes various enzymes and metabolites which inhibit pathogen growth. *Fusarium* is a primary target of Mycostop.

Mycostop is used for the control of seed rot, root and stem rot, and wilt caused by various pathogenic fungi, of container grown ornamentals, herbs, and vegetables. Mycostop has been tested as a seed treatment on various ornamentals, leaf vegetables, root crops, and herbs. It can be mixed with seed in a planter box, or the seeds can be coated by mixing them with Mycostop in a small container. The use of Mycostop as a transplant dip and a soil drench will be discussed in Chapter 7.

5.3.2 Materials and methods

A preliminary seed treatment was performed by placing 200 basil seeds known to have an incidence of 25% *Fo* contamination, in a small glass jar along with a one gram packet of Mycostop®. The jar was shaken until the seeds became coated with the dry powder. Half the seeds were plated onto Komada/*Fusarium* medium; and the other half were placed into sterile petri dishes containing filter paper. Both sets of plates were incubated at 27°C for two days, and were subsequently checked for *Fusarium* contamination and

seed germination, respectively. This experiment was only performed once; because Mycostop works best as a root colonizer, it was concluded that greenhouse grow-out tests of plants treated with Mycostop and inoculated with *Fob* might give a better indication of treatment results. A combination of seed treatments and soil drenches was also undertaken. Those experiments are discussed further in Chapters 6 and 7, respectively.

5.3.3 Results and discussion

Seventy-seven percent of the seeds germinated, although 15 of those seeds exhibited browning of the leaves, stem, and/or roots. Ten percent of the seeds plated on Komada medium were contaminated with *Fusarium* (data not shown), which is a notable reduction considering the initially high level of contamination. However, 10% contamination is unacceptably high for purposes stated earlier.

5.4 Bleach (Sodium Hypochlorite) Treatments

5.4.1 Literature review

Chlorine bleach (sodium hypochlorite) is often utilized as a surface disinfestant for seeds and other plant parts. It is recommended for eradicating fusaria from seed surfaces (Damicone et al., 1981); however, if the pathogen resides

partially or totally within the seed, bleach treatments will not be effective. For example, bleach treatments were able to reduce but not completely exclude *Fusarium moniliforme* and *F. oxysporum* from asparagus seed (Damicone et al., 1981; Elmer and Stephens, 1988) or *F. moniliforme* from corn seed (Daniels, 1983). In tandem with a treatment which is able to kill an internal seed pathogen, such as a systemic fungicide/acetone treatment or a hot water treatment, bleach treatments work quite effectively (Elmer and Stephens, 1988).

Several different concentrations and soaking time periods have been utilized by various researchers. Damicone et al. (1981) soaked asparagus seeds in 10% Clorox™ (0.525% NaOCl) for ten minutes. The treatment reduced *F. oxysporum* contamination by nearly two-thirds, without affecting germination rates. Daniels (1983), attempting the same treatment on corn seeds, found a 75% reduction in seed infestation and a 65% reduction in seedling damage caused by *F. moniliforme*. Elmer and Stephens (1988), on the other hand, were able to reduce fungal contamination from 73% to 2%, with a 30 minute soak in 20% Clorox™ with 3 drops of Tween 20. Chun and Schneider (1994) stated that buffering a

5.4.3 Results and discussion

The results of the bleach treatments are presented in Table 5.1. No bleach treatment was able to completely eliminate *Fusarium*; however, the reduction of contamination was always significant. Germination rates were never significantly affected by any of the bleach treatments. Nevertheless, a bleach treatment alone is not a viable answer to the control of *Fob* on basil seeds; however, in tandem with another treatment method, it may be a successful assistant.

The results also suggest that *Fob* is mostly a surface contaminant of basil seed, but some basil seed may harbor internal infections as well.

5.5 Hot Water Treatments

5.5.1 Literature review

The control of plant pathogens through heat treatment has been practiced for over 100 years. Applied through water, air, or vapor, this method consists of heat treatment of seeds or plant parts at temperature and time regimes which kill the selected pathogen, without significant injury to the host. The benefits and disadvantages of hot water treatments on seeds or other plant material were summarized

2.6% NaOCl solution to pH 7, from pH 11.6, significantly reduced rice seed contamination by fungi and bacteria.

5.4.2 Materials and methods

Four treatments, utilizing changes in NaOCl concentration, soaking times, and pH were attempted. The treatments were:

a. 20 minute soak in 10% Clorox™ (0.525% NaOCl);

b. 1 hour soak in 10% Clorox;

c. 2 hour soak in 10% Clorox; and

d. 20 minute soak in 10% Clorox buffered to pH 7 with 1 N HCl.

Two hundred seeds known to exhibit 25% *Fo* contamination were utilized per treatment. The seeds were soaked in their respective solutions for the corresponding time period. After treatment, the seeds were rinsed in sterile distilled water. Half the seeds from each treatment were plated onto Komada medium and the other half were plated onto filter papers inserted into sterilized glass petri dishes. A control (untreated seed) treatment was also included. The plates were incubated at 27°C for three days, after which they were checked daily for seed germination and *Fusarium* contamination.

by Baker (1962). The consequences of extreme heat on living organisms include enzyme denaturation, the melting of fats, destruction of hormones, tissue asphyxiation, and depletion of food reserves. For a hot water treatment to be successful, the plant pathogen must be more sensitive to heat than its host plant. This is possible due to the nature of the enzyme systems of parasitic microorganisms, which are generally more specialized than those of saprophytes. The thermal death point of the host plant must be considerably higher than that of the parasite. The heat tolerance of seed depends on the seed's moisture content, the age and vigor of the seed, the size of the seed being treated, the physical condition of the seed, and the varietal susceptibility of the cultivar. Problems with hot water treatments include asphyxiation of host tissues, premature germination, or -- a predicament with basil seeds -- the exudation of mucilaginous material which sticks the seed together upon drying. It is also unknown if seeds which exude mucilage are able to be soaked in water and still be viable for planting at a later date, or if planting immediately after treatment is required.

The recommended heating interval for seeds smaller than 0.6 cm is 30 minutes. Presoaking the seed in cold water for four to twelve hours is practiced to eliminate air between the seed coat and the internal portion of the seed, because water is a better heat conductor than air. The pathogen may also be stimulated to grow during this presoak, rendering it more susceptible to the heat treatments. It is best to use water/tissue ratios exceeding 100:1 by weight, for treatments of 30 minutes or less (Baker, 1962).

5.5.2 Materials and methods

The thermal death point temperatures for both basil seed and *Fob* were determined by adding fifty seeds known to be contaminated with *Fob* to a glass test tube containing 4 mls of distilled water at room temperature. At least two test tubes were utilized per temperature treatment. The seeds were presoaked for at least four hours in the water before heating. The test tubes were placed in a preheated test tube heater (Barnstead/Thermolyne Modular Dri-Bath, Model No. DB16525) and heated for 20 minutes at the desired temperature. The test temperatures were 50°C (122°F) and 60°C (140°F). A room temperature treatment was utilized as a control. After the soak, the test tube contents were

poured into a sieve to drain out the water. The seeds in the sieve were rinsed with cold, distilled water, then dried on sterile filter paper under the hood. Germination was examined by placing seeds in a glass petri dish (100 x 15 mm) with a moistened sterile filter paper inside (Fisherbrand filter paper, diameter 9.0 cm). The plates were checked after 3 - 4 days for seed germination.

Germination was reduced by more than 50% at 60°C. The germination potential at 50°C and at room temperature were equal; both exhibited about 97% germination. Based on these results, tests were carried out at 54°C (129.2°F); 56°C (132.8°F); 58°C (136.4°F); and 60°C (140.0°F), along with a room temperature (unheated) control. The treatments were carried out as stated above; except to check *Fusarium* contamination, half of the treated seeds were plated onto Komada medium (25 basil seeds per 100 x 15 mm petri dish). The plates were inspected for *Fusarium* growth after 3 - 4 days. The experiment was performed four times.

A test was also carried out to discover if hot water treatment of basil seed would result in a loss of germination after 24 hours or one week or storage. Two hundred basil seeds were soaked in distilled water for four

hours. They were then dried under the sterile hood for one hour, on sterilized filter paper. The seeds were incubated at room temperature under the sterile hood, half for 24 hours and half for one week. Afterward, the seeds were removed from the paper with a forceps and plated onto wetted sterile filter paper inside glass petri dishes. The dishes were incubated at 27°C for at least one week.

5.5.3 Results and discussion

There were no significant differences in germination between the room temperature control and the 54°C or 56°C treatments (Table 5.2). Germination was decreased by 20.25% by the 58°C treatment, and by 42.5% by the 60°C treatment. Variation between replications ranged from 17% to 65% under the 60°C treatment.

The hot water treatments significantly reduced *Fob* contamination (Table 5.3); however, the fungus was not completely eliminated from the seed until the water temperature reached 60°C. Since this lot of basil seed also demonstrated high rates of heat kill at this temperature, this indicates that the thermal death point of basil seed is very close to the thermal death point of *Fob*. *Fusarium oxysporum* is said to be one of the most heat resistant fungi

(Hoitink and Fahy, 1986). It is also one of the more long-lived soilborne fungi, making *Fusarium oxysporum* one of the more difficult plant pathogens to control.

The mucilaginous later secreted from the basil seeds dried quickly on the filter paper. When the seeds were removed from the filter paper after 24 hours, the dried mucilage caused them to stick to the paper, creating some difficulty in removing the seeds. At least fourteen seeds shattered during attempts at removal with the forceps. Out of 86 seeds removed from the filter paper, 64 germinated in the glass petri dishes after a 24 hour drying period. Several of the seeds exhibited contamination with *Fusarium* and *Aspergillus*, however. Seventy-six of 100 seeds left to dry for a week germinated. It can be concluded that germination was not impaired by drying the seeds; however, a more suitable substrate for drying and perhaps of seed removal needs to be devised.

5.6 Bleach/Hot Water Combination

5.6.1 Literature review

Since sodium hypochlorite (bleach) is utilized as a surface sterilizant, and hot water treatments are successful against internally seedborne pathogens, a combination of the

two practices should achieve what each treatment separately cannot accomplish against a seedborne pathogen which may live both inside and outside the seed.

No studies were found which utilized both of these strategies together; however, several researchers have utilized chemical treatments in tandem with a hot water treatment (Grondeau and Samson, 1994). Elmer and Stephens (1988) followed a 30 minute, 20% bleach soak with a 24 hour benomyl (25,000 ppm)/acetone treatment on asparagus seeds, with success; fungal contamination was eliminated completely, with no reduction in germination. Reversing the treatment order (that is, soaking in benomyl/acetone prior to the bleach treatment) did not alter the results.

5.6.2 Materials and methods

One hundred basil seeds with a 25% incidence of *Fo* contamination were placed into a 50 ml glass beaker. A solution of 10% Clorox™ (0.525% NaOCl) was added to the beaker, and the seeds were left to soak for ten minutes. The seeds were rinsed with sterile, distilled water several times before placing them into glass test tubes containing 4 mls of distilled water. The tubes were placed in a preheated test tube heater (see section 5.5.2) for 20

minutes, at a temperature of 55°C. After heating, the seeds were removed, dried under the sterile hood on pieces of sterile filter paper, and arranged onto petri dishes containing Komada/*Fusarium* agar. A control was prepared with one hundred nontreated seeds for comparison. The plates were incubated at 27°C and checked daily for germination and *Fusarium* contamination. The experiment was replicated three times.

5.6.3 Results and discussion

No *Fusarium* was cultured from any of the three hundred treated seeds, as compared to the control, which exhibited 20% *Fusarium* contamination. Seed germination decreased after the bleach/hot water treatment, to 68%, as compared to 90% germination for the control.

The bleach/hot water combination was the most successful of all the seed treatments attempted in this study. It is likely that since neither treatment alone was able to completely eliminate *Fob* from seed, that most infected basil seed harbors the fungus both internally and externally; although as indicated by the significant decontamination of the seed by the bleach treatment alone, *Fob* is mainly an external contaminant of basil seed. The

amount of internal infection, although modest, warrants the implementation of a hot water treatment as well.

Table 5.1 Sodium hypochlorite treatments: percent germination and *Fusarium* contamination of basil seed.

	Unt.	10%/20	10%/60	10%/120	10%/20/B
Germination	75a[a]	75a	78a	69a	69a
Fusarium	21a	3c	1e	2d	5b

[a] Means in a row for each test followed by the same letter are not significantly different (P=0.05).

Key to treatments: **Unt.**: Untreated; **10%/20:** 20 minute soak in 0.525% NaOCl; **10%/60:** 60 minute soak in 0.525% NaOCl; **10%/120:** 120 minute soak in 0.525% NaOCl; **10%/20/B:** 20 minute soak in 0.525% NaOCl buffered to pH 7 with 1 N HCl.

Table 5.2 Hot water treatments: germination of basil seed.

	Germination (%)				
	Unt.[a]	54°C	56°C	58°C	60°C
Rep.1[b]	91	79	67	73	49
Rep.2	76	59	67	56	17
Rep.3	91	83	72	53	65
Rep.4	86	98	89	81	43
Ave.[c]	86a	79.75a	73.75a	65.75ab	43.50b

[a]Unt. = untreated (room temperature treatment)
[b]Rep. = replication. Each replication is an average of 200 seeds.
[c]Ave. = average. Means with the same letter are not significantly different at P = 0.01.

Table 5.3 Hot water treatments: elimination of *Fusarium oxysporum* f. sp. *basilicum* from basil seed.

	Contamination (%)				
	Unt.[a]	54°C	56°C	58°C	60°C
Rep.1[b]	27	0	0	1	0
Rep.2	20	7	0	0	0
Rep.3	28	3	0	0	0
Rep.4	22	1	1	0	0
Ave.[c]	24.25a	2.75b	0.25b	0.25b	0b

[a]Unt. = untreated (room temperature treatment)
[b]Rep. = replication. Each replication is an average of 200 seeds.
[c]Ave. = average. Means with the same letter are not significantly different at P = 0.01.

CHAPTER 6
GREENHOUSE GROW-OUT TESTS OF SEED TREATMENTS

6.1 Literature Review

Seed germination and contamination tests are typically carried out on blotter paper, in glass petri dishes on sterile filter paper, or in petri dishes containing an agar medium. These methods are quick and easy ways to assess germination; however, it is not necessarily true that a germinated seed appearing to be pathogen-free will grow up to be a healthy plant. Daniels (1983) found that *Fusarium moniliforme* was detected in aseptically germinated corn seedlings after the seed had been treated with various surface sterilization techniques, even when less or no infestation had been evident in corn seed plated onto selective media. Few of the corn seeds showed infestation by *F. moniliforme* when plated on Komada agar; however, the fungus was recovered from 10 - 75% of the seedlings from treated seeds.

Greenhouse grow-out tests assess more than simply the germination potential of treated seed. It is necessary to determine the point in a plant's life cycle at which it is most susceptible to pathogen attack. The opportunity is created to follow the plant throughout the life cycle,

taking into account the effects of soil microbiology, watering and fertilizer regimes, etc. upon the effectiveness of the seed treatment in reducing or eliminating the seedborne pathogen.

This study was performed to determine if *Fusarium* wilt occurred in plants after the seeds were subjected to various treatments, and at what point in the life cycle any wilt symptoms occurred. Concurrently, half of the seeds from each seed treatment were plated onto Komada medium to compare the number of seeds showing *Fo* contamination with the number of plants exhibiting wilt symptoms.

6.2 Materials and Methods

Four seed treatments were utilized: coating the basil seed with Mycostop powder (Chapter 5, section 3); coating the seed with Mycostop, then amending the soil containing the seeds with a soil drench of 0.1% Mycostop every three weeks; soaking the seed in hot water (58°C and 59°C) for 20 minutes; and soaking the seed in a 10% solution of Clorox™ (0.525% NaOCl) for ten minutes. Two hundred seeds were used for each seed treatment. All treatments were replicated twice, except for the Mycostop seed treatment/soil drench combination, which was only performed once.

Seeds treated with Mycostop were placed in a small jar with a one gram packet of Mycostop powder and shaken until the dry seeds were coated with the powder. For the Mycostop seed treatment/soil drench treatment, the soil was amended three weeks after the seed treatment with one liter of 0.1% Mycostop. Details regarding soil drench treatments are given in Chapter 7. Under the hot water treatment, basil seeds were soaked in test tubes containing distilled water for four hours at room temperature, before heating. The test tubes containing the seeds were placed into the Dri-Bath which had been heated to 58°C (for the first trial) or 59°C (for the second trial). After 20 minutes, the test tubes were drained of water through a sieve, and the seeds were rinsed with cold distilled water. The seeds were removed from the sieve and dried under a transfer hood on sterile filter paper. The seeds treated with Clorox™ were soaked in a glass beaker containing ten mls of 0.525% NaOCl amended with a drop of Tween, for 10 minutes. The beaker was drained, and the seeds rinsed with distilled water over a sieve. The seeds were removed and left to dry under a transfer hood on sterile filter paper. A control treatment was left untreated.

Half the seeds from each treatment were plated onto Komada agar for the first replication. The remaining seeds were planted in a seedling tray containing 200 3 x 3 cm cubes, amended with Metro Mix 350. The tray was placed in a mist room in the greenhouse. Every five minutes, the tray received a spray of water in mist form, lasting for 10 seconds. The temperature in the mist room was kept at approximately 30°C (86°F). Seed germination was noted, as was the incidence of *Fo* colonies on the Komada plates. After five weeks of growth, some plants were transferred singly to 12 cm pots, and any wilt symptoms were noted. The stems from plants with wilt symptoms were removed and plated onto Komada agar, to check for the presence of *Fo*.

6.3 Results and Discussion

The results are presented in Table 6.1. Germination was not significantly decreased by any treatment except for the 59°C hot water treatment. The 59°C treatment was rendered for the second trial to determine if the higher temperature would decrease the number of wilted plants occurring under the 58°C seed treatment, without decreasing the germination capability. Only one seed out of 100

germinated at 59°C; proving the thermal death point of this basil seed lot was approximately between 58 and 59°C.

All the seed treatments exhibited less contamination with *Fo* than the control treatment, as evidenced by the colonies on the Komada plates. Only the seeds treated with Mycostop had a high number of seeds with *Fo*, as compared to the control. However, as stated in section 5.3.3, the results shown on the Komada plates are not really an accurate representation of Mycostop's possible activity against *Fusarium*, since one of the primary actions the soil bacterium utilizes against a pathogen is the colonization of plant roots in advance of the fungus. The bleach treated seeds possessed only one contaminated seed; whereas the hot water treated seed (58°C) had no *Fo*.

All the seed treatments included some plants producing wilt symptoms. The symptoms generally began to appear when the plants were between six and seven weeks old. All stems from wilted plants which were plated onto Komada displayed *Fo* growth, verifying that the wilt was caused by *Fob*. The only treatment displaying a reduced number of wilted plants, as compared to the control, was the hot water treatment (58°C). Only 55 of the hot water treated seeds developed

into basil plants, with four of those plants (7.3%) displaying wilt symptoms. The other three treatments exhibited close to equal or more wilted plants than the control. The seeds treated with Clorox™ manifested a surprisingly high number of plants with wilt symptoms. An average of 49.8% of the basil plants grown from seed treated with bleach had *Fusarium* wilt. The reason for this outcome is unknown, though perhaps the bleach effectively eliminated most of the microbes from the seed surface, making the seed more vulnerable to attack by *Fob*.

These findings confirm Daniels' observation that the germination of seeds in a petri dish containing either agar media or a wetted sterile filter paper, is a less sensitive method of rating seedborne contamination as it relates to subsequent plant disease, than is the observation of the plants themselves. The results also show that *Fob* can remain in the seed and cause subsequent damage to basil plants, rather than merely killing the seeds or small seedlings themselves. This is consistent with the mechanisms of vascular wilt diseases, which normally manifest themselves while the plant is developing. It also suggests *Fob* is systemically transmitted through the plant from the

seed, as the plants display wilt symptoms at various stages throughout their life cycle.

Table 6.1 Results of greenhouse grow-out tests for four basil seed treatments executed to eliminate *Fusarium oxysporum* f. sp. *basilicum*.

Tmt[a]	Control		Hot water (58°)		Bleach (20m/10%)		Mycostop (seed)		Mycostop (seed and soil drench)[b]
	T1	T2	T1	T2	T1	T2	T1	T2	T1
Germ (%)[c]	79	85	55	1	57	68	63	87	79
Kda[d]	20	/	0	/	1	/	12	/	/
wilt	18	20	4	0	35	26	10	17	20
% wt.[e]	22.8	23.5	7.3	0	61.4	38.2	15.9	19.5	25.3

[a] Tmt = treatment.

[b] The soil drench was carried out every three weeks, at 0.1% Mycostop (1 gram Mycostop powder per liter distilled water). Fifty mls were added to each plant.

[c] Germ (%) = percent germination.

[d] Kda = number of seeds with *Fusarium oxysporum* colonies on Komada agar. One hundred seeds per treatment were placed onto Komada agar.

[e] % wt = the percentage of wilted plants as compared to the number of germinated plants.

CHAPTER 7
THE USE OF MYCOSTOP AS A SOIL DRENCH IN POTTING MIXES CONTAMINATED WITH *FUSARIUM OXYSPORUM* F. SP. *BASILICUM*

7.1 Literature Review

As explained in Chapter 5, Mycostop® is a fungal biocontrol agent consisting of the soil bacterium, *Streptomyces griseoviridis*. It is commercially available for the control of seed rot, root and stem rot, and wilt caused by various pathogenic fungi. The agent acts by depriving pathogenic fungi, particularly that of *Fusarium* species, of living space and nourishment by colonizing plant roots before the pathogen. It is also able to secrete various enzymes and metabolites that inhibit pathogen growth.

Seed treatments with Mycostop were not successful in reducing or eliminating *Fusarium* wilt from basil, as established in Chapters 5 and 6. Mycostop has been successfully utilized on plants other than basil as a soil drench and a transplant dip. Wick and Haviland (unpublished data) attempted soil drenches and transplant dips with Mycostop on basil plants. Both treatments were unsuccessful in protecting basil from *Fob*. However, inoculum density of

the pathogen was relatively high (approximately 10^5 *Fob* propagules/ml).

Keinath (1994) also drenched soil containing basil plants with 0.01% and 0.1% solutions of Mycostop three days before inoculating the plants with a 10^6 microconidia per ml suspension of *Fob*. The disease was not controlled at all by the lower concentration of Mycostop, but the higher concentration reduced wilt symptoms by 40%.

In this study, Mycostop was added to soil five days preceding the addition of *Fob*. A *Fob* concentration of 3.5 x 10^3 propagules/gm soil -- about half of what was utilized in the previously mentioned experiments -- was used. The study was done to ascertain if a) the soil bacterium needed more than three days to colonize the basil roots in order to outcompete *Fob;* and b) Mycostop was able to delay or lessen the severity of any wilt symptoms as compared to the inoculated plants without Mycostop. Greenhouse producers of basil have stated that they would be willing to live with *Fusarium* wilt if there was a management strategy they could establish which would enable them to harvest plants early in order to avoid the disease.

7.2 Materials and Methods

Two hundred Genovese basil seeds from a seed source apparently free of *Fob* contamination were treated with Mycostop by adding a one gram package of dry Mycostop powder to a jar, adding the seeds, and shaking the jar so as to coat the seeds with the Mycostop powder. The seeds were planted in a seedling tray containing 200 3 x 3 cm squares, and left to germinate in the greenhouse. An additional two hundred basil seeds from the same seed source were soaked in distilled water at 58°C (see Chapter 5, section 5) and were planted in a seedling tray in the greenhouse. Two weeks later, one hundred basil seedlings of each seed treatment type were transplanted into 12 cm pots. The seedlings treated with Mycostop as seeds were again treated with a soil drench of Mycostop. An 0.1% solution, containing one gram of Mycostop powder suspended in one liter of distilled water, was prepared according to the directions on the label. Each of the 100 plants received 50 mls of the solution. The plants germinated from the hot water treated seeds received 50 mls each of distilled water. All plants were watered two and one half hours before and again two hours after the Mycostop application.

Earlier, the *Fob* inoculum source was prepared to add to the basil plants. Fifty grams of bentgrass seed and 25 mls of distilled water were autoclaved twice for one hour each time. A suspension of *Fob* isolate Misc28 was added to the autoclaved seed. Two weeks later, the inoculated seed was placed in an autoclaved paper bag, which was turned over every day for two weeks. The contents of the bag were then ground in a Wiley mill using a #40 screen, then stored until use in a 3°C refrigerator. The mixture was found to contain 3.5×10^5 *Fob* propagules per one gram ground bentgrass seed. Four days after the Mycostop treatments, two hundred quart-sized Ziploc plastic bags were each filled with 10 grams of Metro Mix 350. One hundred of the bags also received 0.1 grams of the inoculated ground bentgrass seed. This ensured that approximately 35,000 *Fob* propagules would be added to each plant. Since the inoculum was added in ten grams of soil, 3.5×10^3 *Fob* propagules was the inoculum level added to each plant. The other one hundred bags received 0.1 grams of autoclaved ground bentgrass seed. The next day, five days after Mycostop treatment, fifty plants that had been treated with Mycostop, and fifty that had not, received the 0.1 grams inoculated seed/10 grams soil mix. The mix

was added to each plant by removing the plant from the pot, spilling the remaining soil onto a sheet of brown paper, pouring the inoculum mix into the soil, and rolling the paper about so that the mix blended with the soil. The inoculated soil was then added back to the pot, along with the basil plant. The other one hundred plants -- fifty which had received the Mycostop treatment and fifty which had not -- received the 0.1 grams autoclaved seed/10 grams soil mix in the same manner. Each plant was then watered thoroughly. The plants were numbered to correspond with each of the four treatments, then distributed on the greenhouse bench. The experiment was set up as a randomized block design with five blocks per treatment and ten plants per block. The treatments were:

plants 1 - 50: No Mycostop/Autoclaved seed.

plants 51 - 100: Mycostop/Autoclaved seed.

plants 101 - 150: Mycostop/*Fob*-inoculated seed.

plants 151 - 200: No Mycostop/*Fob*-inoculated seed.

The appearance of wilt symptoms was recorded every two or three days. Three weeks after *Fob* inoculation, the number of plants from each block exhibiting wilt symptoms was recorded.

The data was analyzed by analysis of variance (ANOVA), using Statistix 4.1 statistical software (Table 7.2).

7.3 Results and Discussion

The results are presented in Table 7.1. The total number of wilted plants was nearly equal for each inoculated treatment. There were no noninoculated plants displaying wilt symptoms. There was no significant difference between the blocks. Wilt was observed within 11 days after *Fob* inoculation. Two weeks after *Fob* inoculation, 30% of the *Fob*-inoculated, Mycostop-treated plants were wilted, as were 42% of the *Fob*-inoculated, nontreated plants.

It does not appear that Mycostop delays symptom expression at all, or that it offers significant protection against *Fob*. Field producers of basil leaves and seed, as well as hydroponic basil growers, do not have the luxury of allowing even a small amount of pathogenic material to be present in their field or system. *Fusarium* is long-lived in the soil and thereby has the potential to infect subsequent generations of basil plants. *Fob* conidia flow freely throughout a hydroponic system, as well as through the air, and can therefore spread the disease to many, if not all, basil plants in the greenhouse. As affirmed in Chapter 2,

asymptomatic plants which harbor *Fob* can spread the pathogen through their seed. Therefore, Mycostop would not be an effective treatment in these situations, which require complete eradication of *Fob*.

Table 7.1 Data from the four treatments and five blocks per treatment involving the control of *Fusarium* wilt of basil through Mycostop® soil drench treatments.

	Treatments				
	Myco NF[a]	Myco FO[a]	NT NF	NT FO	Total
Block 1	0[b]	7	0	6	13
Block 2	0	6	0	6	12
Block 3	0	6	0	7	13
Block 4	0	6	0	8	14
Block 5	0	5	0	4	9
Total	0	30	0	31	61

[a] NF = not inoculated with *Fob*. FO = inoculated with *Fob*.
[b] Number of wilted plants per block.

Table 7.2 Analysis of variance table comparing differences between the four treatments and the five blocks per treatment.

Source	df	SS	MS	F
Treatments	3	186.15	62.05	104.81**
Blocks	4	3.70	0.925	1.5625
Error	12	7.10	0.592	
Total	19			

**There are significant differences between treatments at the $P < 0.01$ level. There were no differences between blocks ($f_{4,12\ 0.05} = 3.26$).

CHAPTER 8
A SURVEY OF DISEASE SUPPRESSIVE COMPOSTED POTTING MIXES FOR THEIR ABILITY TO SUPPRESS *FUSARIUM* WILT OF BASIL

8.1 Literature Review

The disposal of wastes into water sources, into landfills, or by incineration has been known to cause serious environmental problems. These problems have incited scientists to examine alternatives to these disposal methods (Hoitink and Fahy, 1986). It has been determined that composting organic wastes can not only assist in decreasing pollution, but can benefit many diverse markets, including the turf, greenhouse, and agricultural industries.

Composts can assist in water retention, pH balance, and soil enrichment. Some composts have also demonstrated plant disease suppression properties. Potting mixes containing composts have been touted as partial or total replacements for fungicides (Hoitink et al., 1991). Previously, pot mixes for the production of greenhouse and nursery crops had been steam-sterilized or fumigated to kill plant pathogens (Hoitink et al., 1976).

Quality control of these composts can be difficult. Composts were formerly considered to be too inconstant for use in disease suppression (Hoitink et al., 1976). However,

researchers are slowly discovering the biological, chemical, and physical characteristics of a suppressive compost, and how to manipulate these attributes for marketing purposes.

A suppressive medium is a mixture that sustains plant growth, but due to its physical, chemical, or microbial composition does not support plant diseases (Quarles and Grossman, 1995). Physical factors leading to the elimination or suppression of plant pathogens include heat killing, and the structure and size of the compost particles relating to drainage and aeration (Hoitink and Fahy, 1986; Quarles and Grossman, 1995).

Chemical factors affecting suppression include the carbon/nitrogen ratio of the compost, as well as the nitrogen form present. The carbon/nitrogen (C/N) ratio affects the suppression and the conduciveness of soilborne pathogens such as *Fusarium*. High C/N ratio materials such as tree barks suppress *Fusarium* diseases, whereas low C/N ratio compost, such as that prepared from municipal sewage sludges, aggravate *Fusarium* wilt diseases. This is due to the fact that sludges tend to release ammonium, and high ammonium and low nitrate nutrition exacerbates Fusarium wilt. Bark composts immobilize ammonium, therefore, the

nitrogen available for plant growth is predominantly nitrate (Hoitink et al., 1991; Quarles and Grossman, 1995).

The suppression of disease through biological processes depends largely upon the recolonization of biocontrol agents into composts during the curing stage of composting. The longer the curing stage, the more diverse the recolonizing microflora will be. Scientists have found ways to manually introduce specific antagonists into composts before significant recolonization occurs during the curing period (Grebus et al., 1994; Hoitink et al., 1994; Quarles and Grossman, 1995). This is performed to decrease variability among the microbial species which colonize the compost and suppress plant pathogens.

There are two biological control mechanism which can occur in compost-based substances: general suppression and specific suppression (Hoitink et al., 1991; Quarles and Grossman, 1995). General suppression occurs when the compost microflora either compete directly with the plant pathogen for nutrients, or prevent spore germination or host infection through microbiostasis. This is the explanation for suppression of *Pythium* and some *Phytophthora* diseases. Controlling pathogens such as *Rhizoctonia* or *Fusarium*

involves an entirely different biological control mechanism. Specific suppression is due to specific microbial antagonists which colonize the compost during the curing phase. Only a very few microorganisms are capable of suppressing *Rhizoctonia* and *Fusarium*, the most important of which are *Trichoderma hamatum*, *T. harzianum*, and *Flavobacterium balustinum* (Grebus et al., 1993). These biocontrol agents may not consistently recolonize composts after heating; therefore, they may have to be applied manually. Combinations of antagonists, i.e. *T. hamatum* (*Th*) and *F. balustinum* (*Fb*) are more effective together in the suppression of *Rhizoctonia* and *Fusarium*, rather than separately. This was shown by Trillas-Gay et al. (1986), who found *Fusarium* wilt of radish was better reduced by the combination of *Th* and *Fb*; and by Grebus et al. (1993), who was able to lessen *Rhizoctonia* damping-off and *Fusarium* wilt of radish with *Th* and *Fb*.

The effect of composted materials on *Fusarium* wilt diseases has not been clearly documented. As mentioned above, Trillas-Gay et al. (1986) stated the suppressive effect of composted hardwood bark to *F. oxysporum* f. sp. *conglutinans* was biotic in nature, and that *Th* and *Fb* were

consistently effective against *Fusarium* wilt of radish as long as they were applied together. Kai et al. (1990) found acetone extracts of the bacterial microflora of hemlock bark compost were active against *F. o.* f. sp. *cucumerinum*. Pera and Calvet (1989) reported *Fusarium* wilt of carnation, caused by *F. o.* f. sp. *dianthi*, was suppressed better using composted olive pomace than with composted pine bark or sphagnum peat. Not only was disease incidence decreased with the olive pomace compost, but symptom expression was significantly delayed as well.

In this study, several compost types were evaluated for their activity against *Fusarium* wilt of basil. The objective of the experiment was to discover if any composted media were able to suppress or delay wilt symptoms after the media had been inoculated with *Fob* propagules.

8.2 Materials and Methods

The five types of media utilized in this experiment were: Compost 1, a composted spruce/hemlock potting mixture inoculated with *Trichoderma hamatum* and *Flavobacterium balustinum*; Compost 2, a composted pine bark potting mixture also inoculated with *Th* and *Fb*; Compost 3, an uninoculated compost which is 89% spruce/hemlock bark and 10% peat;

Compost 4, an uninoculated compost consisting of 80% spruce/hemlock bark and 20% peat; and the control was Metro Mix 350, which contains processed bark ash, vermiculite, Canadian sphagnum peat, and washed sand.

One hundred and eighty Genovese basil plants were used for the experiment. There were ten treatments, with ten plants per treatment. The experiment was replicated twice. (Compost 4 treatments were only replicated once, due to the request of the compost manufacturer to test Compost 4 after the first replication was completed.) The ten treatments were: 1) Compost 1, no *Fob;*

2) Compost 1, *Fob;*

3) *Compost 2,* no *Fob;*

4) Compost 2, *Fob;*

5) Compost 3, no *Fob;*

6) Compost 3, *Fob;*

7) Compost 4, no *Fob;*

8) Compost 4, *Fob;*

9) Control, no *Fob;* and

10) Control, *Fob.*

The basil plants were germinated from hot-water treated (a 20 minute soak in 58°C water) seed, from a seed source

believed to be free of *Fob* contamination. The plants were grown in plug trays until they were three weeks old. They were then transferred to 10 cm pots, each with one of the five types of compost media.

Inoculum for the experiment was prepared according to the procedure outlined in Chapter 7. 0.1 grams of ground bentgrass seed inoculated with *Fob* was added to a plastic bag also containing 10 grams by volume of the designated compost type. The two materials were mixed thoroughly. The final inoculum amount added to the plants was 3.5×10^3 *Fob* propagules/gram media. Plants receiving no *Fob* acquired instead 0.1 grams of autoclaved, ground bentgrass seed added to 10 grams of compost in a bag. The contents of each bag were added to the compost containing the basil plants by removing the compost from the pot, pouring it onto a piece of brown paper, and mixing it with the corresponding bag of compost/inoculum combination. The mixture was then added back to the pot, along with the basil plant.

Plant heights were measured, starting the week before inoculum was added, once a week. Wilt was scored on a yes or no basis. The first replication of the experiment was terminated five weeks after *Fob* inoculation. The second

replication was taken down nine weeks after *Fob* inoculation, partially because the winter conditions led to lower sunlight entering the greenhouse, contributing to a decline in basil plant size. Also, it was thought the plants may still exhibit wilt symptoms late in their life cycle, while the plants were aging but still harvestable.

The differences in height between the time of Fob inoculation, and six weeks later (for both trials, and again nine weeks after Fob inoculation for the second trial) were calculated. The results were analyzed using analysis of variance (Statistix 4.1 software). The differences between the means for each treatment were assessed through a least squares distribution (LSD) pairwise comparison of means at P = 0.05.

8.3 Results and Discussion

In experiment 1, five weeks after *Fob* inoculation, the wilt incidences for each compost treatment were: compost 1, 10%; compost 2, 60%; compost 3, 60%; and the control, 80%. None of the plants inoculated with autoclaved bentgrass seed exhibited wilt symptoms. Wilt symptoms were first observed three weeks after *Fob* inoculation. The first treatments to display wilt symptoms were compost 2 and the control, which

each had 50% with symptoms. With experiment 2, the wilt incidences were noted six weeks after *Fob* inoculation and again three weeks later. After week 6, wilt incidences were: compost 1, 50%; compost 2, 70%; compost 3, 10%, compost 4, no wilt; and the control, 60%. After week 9, wilt incidences were: compost 1, 90%; compost 2, 70%; compost 3, 90%; compost 4, 80%; and the control, 90%. Wilt symptoms were first apparent four weeks after *Fob* inoculation. This time, compost 1 (40%) and the control treatment (40%) were the only treatments to demonstrate early wilt symptoms.

Height differences between inoculated and noninoculated plants are shown in Figures 8.1, 8.2, and 8.3, and in Tables 8.1, 8.3, and 8.5. In the first experiment (Figure 8.1, Table 8.1), the *Fob*-inoculated treatment of compost 1 had significantly taller plants than both the inoculated and noninoculated plants in compost 3, even though compost 3 was comparatively slow to exhibit wilt symptoms. This compost mix, consisting of 90% spruce/hemlock bark and 10% peat, was very dense, with very little pore space. Plants in this media were noticeably yellow and spindly as if they had

nitrogen deficiency; however, all plants received 15-16-17 fertilizer at the rate of 300 ppm weekly.

The inoculated and noninoculated compost 1 treatments were significantly different in terms of height; in spite the fact that only one plant wilted. The amount of wilt in the inoculated compost 2 treatment, however, was unusual, since it was -- as was compost 1 -- inoculated with the *Th* and *Fb* organisms. The difficulty may have been due to the large pore spaces compost 2 had. The plants in that treatment did not retain water as well as the others. Hoitink et al. (1976) have stated that the problem with most mixes containing bark compost is that they do not hold enough water. Often the plants in the compost 2 treatments would wilt from lack of water, whereas the other treatments would appear healthy. Once the compost 2 plants were watered, they retained their vigor and the leaves again became turgid. This may have weakened the plants inoculated with *Fob* to the point where they became more susceptible to disease than would healthier plants receiving adequate water.

The results of the second experiment were somewhat different. This may be at least partially due to the

environmental conditions each experiment took place under: experiment 1 was carried out during the months of September and October, whereas experiment 2 took place during November, December, and January. The greenhouse temperature was not significantly lower except for several days when the heating system went unchecked and the temperature dropped to below 60°F; however, the photoperiods were much shorter than they were during the first replication, leading to comparatively stunted basil plants.

Height differentials for the second experiment are noted in Figures 8.2 and 8.3, and in Tables 8.3 and 8.5. Figure 8.2 and Table 8.3 cover the period between 1 December and 9 January, six weeks after *Fob* inoculation. Whereas Composts 1 and 2 had the highest noninoculated plant growth heights, composts 3 and 4 -- with one and no plants wilted, respectively -- demonstrated higher rates of growth with the inoculated treatments. However, this phenomenon soon disappeared three weeks later (Figure 8.3, Table 8.5), as all the treatments exhibited severe wilt, thereby decreasing the height averages of all the inoculated treatments.

Further studies need to be carried out on the utility of composted potting mixes upon *Fusarium* wilt of basil. The environmental conditions need to be maintained in a more stable manner in order to truly compare disease suppression by compost. It can be stated, however, that whereas composts 1 and 2 provided better overall basil growth in the uninoculated treatments, the other two composts, 3 and 4, seemed to be able to delay wilt symptom expression, at least temporarily. The reason behind this discovery is unclear, since composts 1 and 2 were inoculated with the biocontrol agents which were theoretically supposed to insulate the plant against *Fob*. Perhaps composts 3 and 4 were naturally colonized with biocontrol agents which temporarily prevented *Fob* from causing wilt. In any case, if it was proven that a compost was able to delay wilt symptoms for a period of time, the finding would be beneficial to the greenhouse industry. Growers could elude some economic loss by harvesting basil plants early, if *Fob* is suspected to be a problem.

Table 8.1 Data from the eight treatments involving the differences in basil plant height between 22 September and 31 October 1995 of plants grown in composted potting mixes inoculated (FO) or not (NF) with *Fusarium oxysporum* f. sp. *basilicum* (*Fob*)

Treatment	Mean	Sample Size	Group Std. Dev.
Compost 1 NF	38.50	10	2.9155
Compost 1 FO	30.70	10	7.9903
Compost 2 NF	37.30	10	5.6085
Compost 2 FO	23.95	10	10.221
Compost 3 NF	30.00	10	6.9081
Compost 3 FO	25.70	10	8.7502
Control NF	36.70	10	8.7089
Control FO	18.95	10	6.0298
Total	30.23	10	7.4562

Table 8.2 One-way analysis of variance for the eight treatments involving the differences of basil plant height from 22 September to 31 October 1995 of plants grown in composted potting mixes inoculated or not with *Fob*.

Source	df	SS	MS	F
Between	7	3477.10	496.73	8.93**
Within	72	4002.85	55.595	
Total	79	7479.95		

**There are significant differences between treatments at the $P < 0.001$ level.

Table 8.3 Data from ten treatments involving the differences in basil plant height between 1 December 1995 and 9 January 1996 of plants grown in composted potting mixes inoculated (FO) or not (NF) with *Fob*.

Treatment	Mean	Sample Size	Group Std. Dev.
Compost 1 NF	12.55	10	2.4546
Compost 1 FO	5.05	10	2.5868
Compost 2 NF	11.45	10	3.5469
Compost 2 FO	6.15	10	3.6059
Compost 3 NF	9.75	10	1.8447
Compost 3 FO	6.35	10	2.7694
Compost 4 NF	9.15	10	2.7993
Compost 4 FO	6.85	10	1.7488
Control NF	9.95	10	2.8132
Control FO	3.95	10	2.6609
Total	8.12	100	2.7434

Table 8.4 One-way analysis of variance for the ten treatments involving the differences of basil plant height from 1 December 1995 to 9 January 1996.

Source	df	SS	MS	F
Between	9	732.21	81.357	10.81**
Within	90	677.35	7.526	
Total	99	1409.56		

**There are significant differences between treatments at the $P < 0.001$ level.

Table 8.5 Data from ten treatments involving the differences in basil plant height between 1 December 1995 and 24 January 1996 of plants grown in composted potting mixes inoculated (FO) or not (NF) with *Fob*.

Treatment	Mean	Sample Size	Group Std.Dev.
Compost 1 NF	26.85	10	3.8301
Compost 1 FO	6.30	10	4.2374
Compost 2 NF	24.25	10	6.7175
Compost 2 FO	8.00	10	6.6542
Compost 3 NF	20.35	10	3.1363
Compost 3 FO	7.45	10	4.2521
Compost 4 NF	21.70	10	7.8747
Compost 4 FO	8.35	10	3.7642
Control NF	23.90	10	4.2804
Control FO	5.10	10	5.7388
Total	15.23	100	5.2667

Table 8.6 One-way analysis of variance for the ten treatments involving the differences of basil plant height from 1 December 1995 to 24 January 1996.

Source	df	SS	MS	F
Between	9	7021.26	780.14	28.13**
Within	90	2496.43	27.74	
Total	99	9517.69		

**There are significant differences between treatments at the P < 0.001 level.

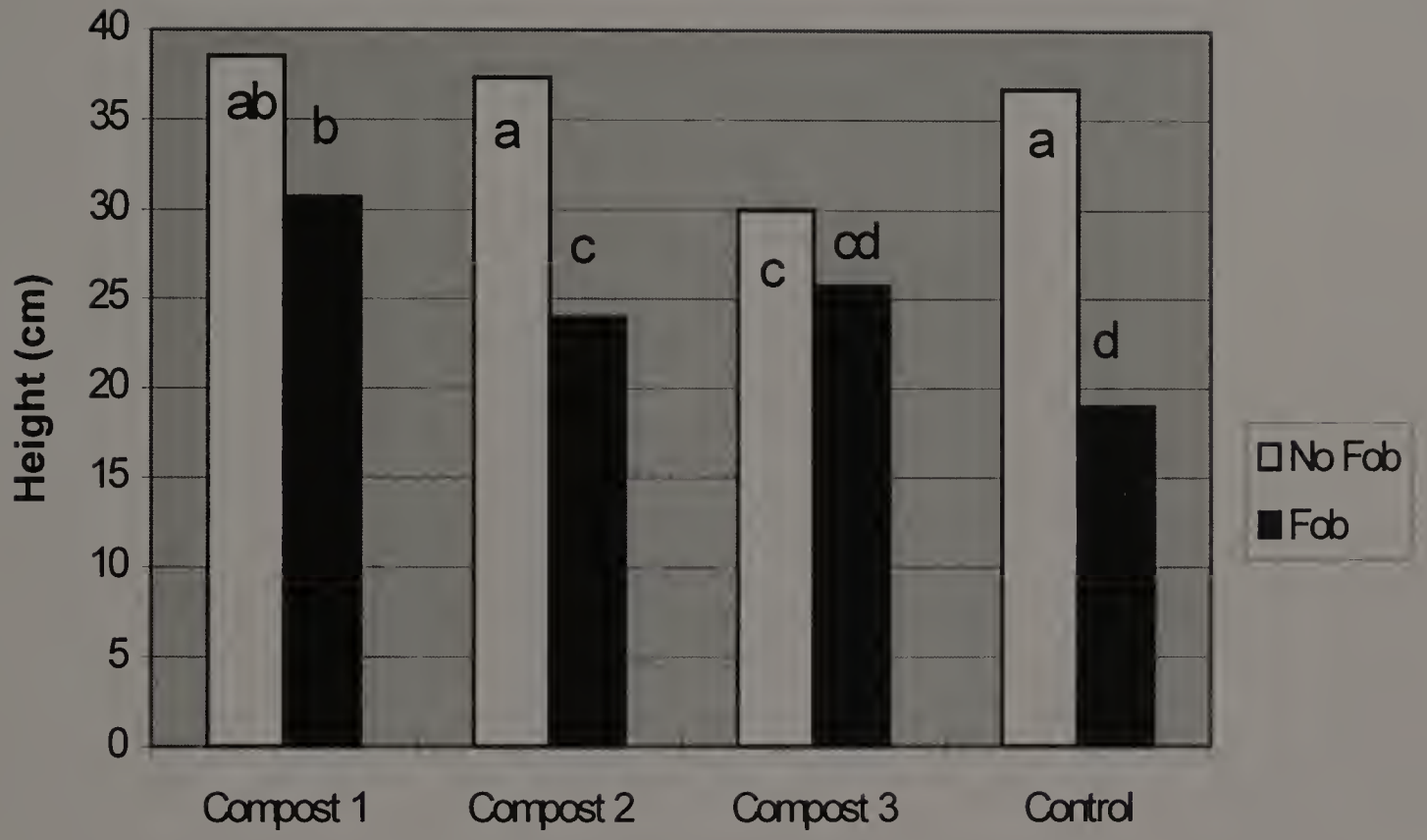

Figure 8.1 Differences in height between 22 September and 31 October 1995 of basil plants in four composted potting mixes inoculated or not with *Fob*. Columns with the same letter are not significantly different at P = 0.05.

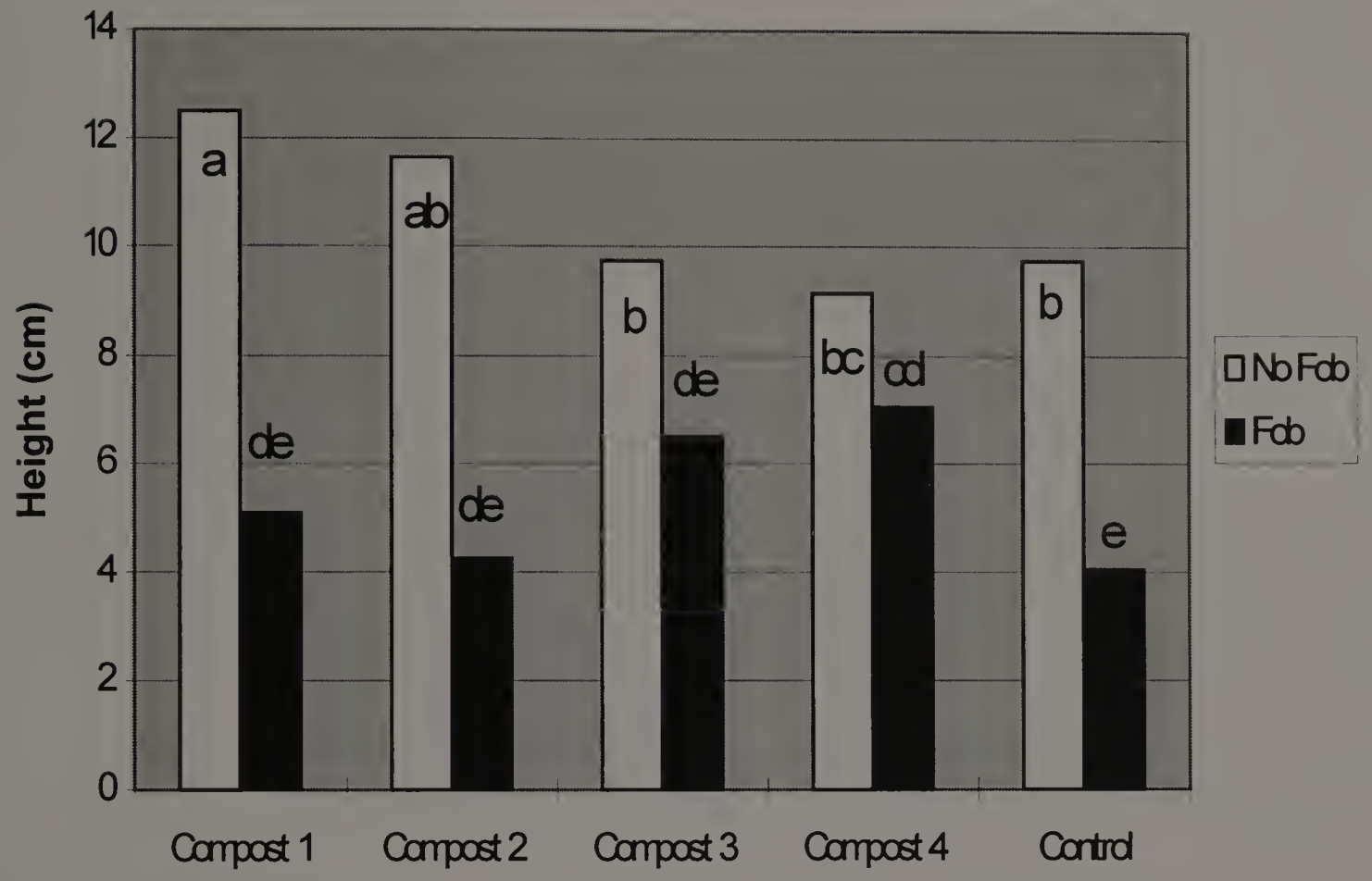

Figure 8.2 Differences in height between 1 December 1995 and 9 January 1996 of basil plants in five composted potting mixes inoculated or not with *Fob*. Columns with the same letter are not significantly different at P = 0.05.

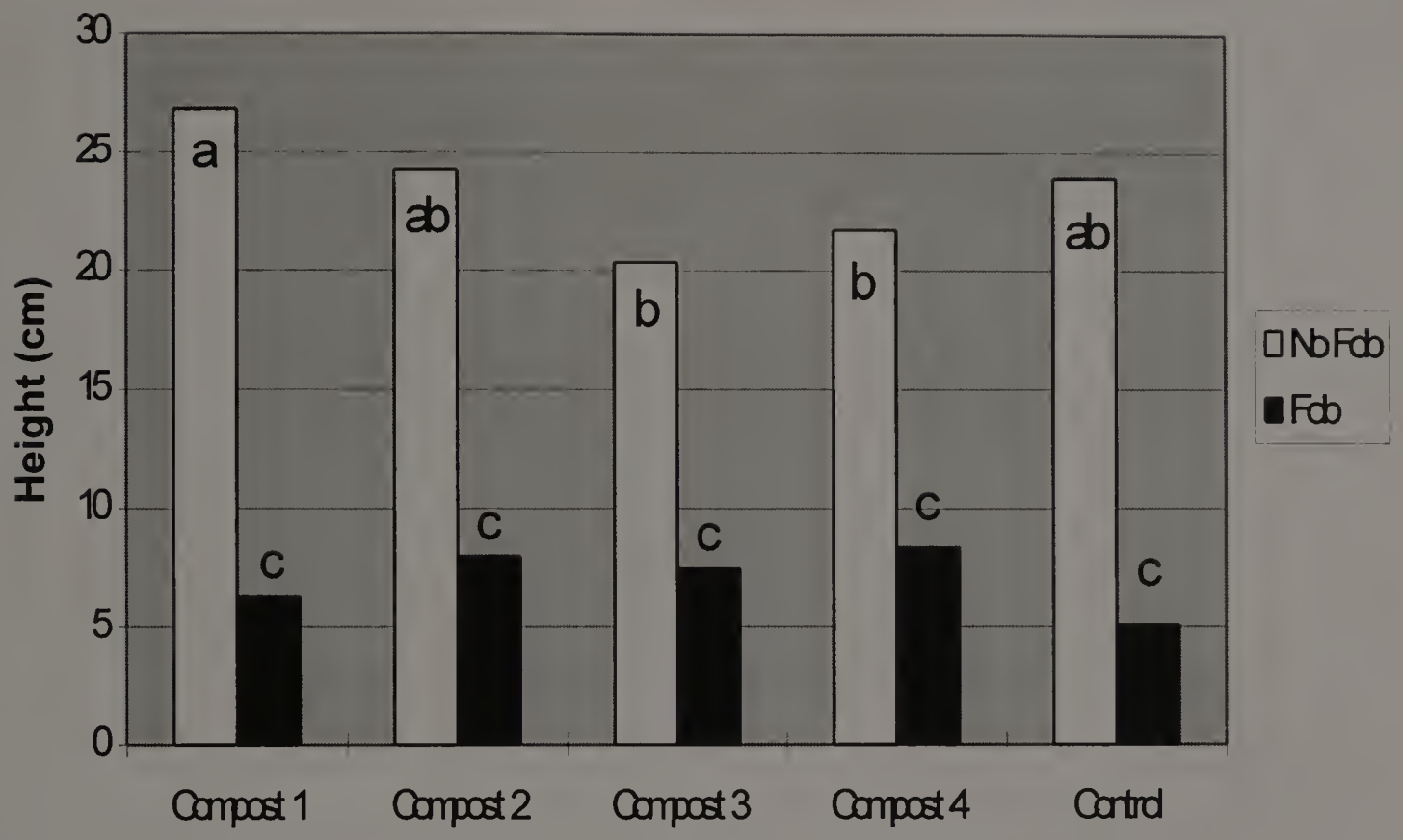

Figure 8.3 Differences in height between 1 December 1995 and 24 January 1996 of basil plants in five composted potting mixes inoculated or not with *Fob*. Columns with the same letter are not significantly different at P = 0.05.

CHAPTER 9
CONCLUSIONS AND FUTURE RESEARCH

Fusarium wilt disease of basil is becoming increasingly common in the United States. The pathogen, a prevailing inhabitant of basil seed, is being distributed throughout the world via the infected seed. Sixteen of twenty-four seed lots tested in this study harbored the causal fungus of *Fusarium* wilt, *Fusarium oxysporum* f. sp. *basilicum* (*Fob*).

Fob is a surface contaminant and perhaps occasionally an internal inhabitant of basil seed. This knowledge is of assistance in the choice of a sufficient seed treatment. The most effective and efficient method of excluding *Fob* from basil seed is the bleach/hot water combination, which eliminates *Fob* completely from basil seed without compromising germination potential. The durable and long-lived nature of *Fusarium* in soil makes it necessary to eliminate *Fob* from plants, soil, and seed, so that subsequent generations of basil plants are protected from the disease.

In this study, no seeds harvested by hand from infected plants harbored pathogenic *Fob*, which leads to the belief that perhaps commercial harvesting of basil seed through threshing is the main pathway to *Fob* contamination. It

remains unclear as to just how *Fob* is transmitted to the basil seed.

The use of VCG's and of RAPD-PCR analysis can assist in identifying a fungal strain as a pathogenic *Fob* isolate. As of this time, RAPD-PCR analysis is not able to distinguish pathogenic and nonpathogenic Fob strains. It seems likely that pathogenic strains belong in a separate VCG from *F. oxysporum* isolates not pathogenic upon basil, which makes heterokaryon tests an instrumental part of the diagnosis of *Fob*, and a viable alternative to pathogenicity testing.

Greenhouse grow-out tests of treated basil seeds have proven to be more sensitive than simple blotter or agar plate tests in terms of verifying *Fob* elimination from basil seed. The mode of movement from seed to plant by *Fob* is likely systemic, since the basil plants display symptoms at various stages of their life cycles.

Mycostop® seed and soil drench treatments are not effective against Fusarium wilt of basil. The use of composted potting mixes inoculated with beneficial biocontrol organisms to grow basil is too inconsistent at this point for recommendation as a control for *Fob*; however,

the mixes show promise in delaying wilt symptoms long enough for at least some harvesting of basil to take place.

Future research projects involving *Fusarium* wilt of basil could include:

--how threshing, to harvest basil seed commercially, affects *Fob* contamination of basil seed;

--the removal of the mucilaginous layer from basil seed to see if it harbors *Fob* spores;

--developing a probe to detect *Fob* inside basil seed; and, --the best strategy for disinfecting basil seeds, utilizing bleach and hot water, commercially.

APPENDIX A
RECIPE FOR KOMADA/*FUSARIUM* MEDIUM

For one liter:

Distilled water	1000.0 ml
$Na_2B_4O_7 \bullet 10H_2O$	1.0 g
K_2HPO_4	1.0 g
KCL	0.5 g
$MgSO_4 \bullet 7H_2O$	0.5 g
Fe-Na-EDTA	0.01 g
D-galactose	20.0 g
L-asparagine	2.0 g
Agar	15.0 g
PCNB (Terraclor 75% WP) (Pentacloronitrobenzene)	1.0 g

Heat to boiling to melt agar. Do not autoclave.
Cool to 50-55°C, then suspend the following in 10 ml distilled water before adding:

Oxgall (Bile Bovine)	0.5 g
Streptomycin sulfate	0.3 g

Acidify the medium to pH 3.8-4.0 with 10% phosphoric acid. It will take about 8 mls. If 85% phosphoric acid is used, it will take about 1 ml.

Note: This medium was developed by Komada as a *Fusarium* selective medium and reported to distinguish *F. oxysporum*, *F. solani*, *F. moniliforme*, and *F. roseum* by the color of the colonies. Galactose is preferentially used by *Fusarium* species.

Streptomycin can be substituted with chloramphenicol at a rate of 0.25 g/liter.

Reference: Komada, H. 1976. A new selective medium for isolating *Fusarium* from natural soil. Proceedings of the American Phytopathological Society 3:221.

APPENDIX B
RECIPE FOR MINIMAL MEDIUM[a]

For one liter:

Distilled water	1000.0 ml
Sucrose	30.0 g
$NaNO_3$[a]	2.0 g
KH_2PO_4	1.0 g
$MgSO_4 \bullet 7H_2O$	0.5 g
KCl	0.5 g
Sterile trace elements solution[b]	0.2 ml
Difco agar	20.0 g

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[a]$NaNO_3$ is used for minimal media containing nitrate (M1). For M2, 0.5 g/L $NaNO_2$ should be added instead of $NaNO_3$. For M3, 0.2 g/L hypoxanthine should be added instead of $NaNO_3$.

[b]Trace elements solution contains the following (per 95 ml distilled H_2O): citric acid, 5 g; $ZnSO_4 \bullet 7H_2O$, 5 g; $FeSO_4 \bullet 7H_2O$, 4.75 g; $Fe(NH_4)_2(SO_4)_2 \bullet 6H_2O$, 1 g; $CuSO_4 \bullet 5H_2O$, 250 mg; $MnSO_4 \bullet H_2O$, 50 mg; H_3BO_3, 50 mg; $Na_2MoO_4 \bullet 2H_2O$, 50 mg.

Reference: Puhalla, J.E. 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. Can. J. Bot. 63:179-183.

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